



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 9/64, C07K 15/06 A61K 37/54, 37/64, C12Q 1/37 // C07K 5/10	A2	(11) International Publication Number: WO 93/00427 (43) International Publication Date: 7 January 1993 (07.01.93)																																																																																																																																											
(21) International Application Number: PCT/EP92/01384 (22) International Filing Date: 19 June 1992 (19.06.92) (30) Priority data: 719,692 24 June 1991 (24.06.91) US (71) Applicant: ERZIEHUNGSDIREKTION OF THE CAN- TON ZURICH [CH/CH]; Walchetor, CH-8090 Zurich (CH). (72) Inventors: SCHWAB, Martin, E.; Waffenplatzstraße 79, CH-8002 Zurich (CH). CARONI, Pierenrico, W.; Fre- iestraße 196a, CH-8032 Zurich (CH). PAGANETTI, Paolo, A.; Birmensdorferstraße 192, CH-8003 Zurich (CH).		(74) Agent: KIRKER & CIE S.A.; P.O. Box 1736, Rue du Mont-Blanc 14, CH-1211 Genève 1 (CH). (81) Designated States: AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i>																																																																																																																																											
(54) Title: NEURITE GROWTH REGULATORY FACTORS																																																																																																																																													
<div style="margin-top: 20px;"> <p style="text-align: right;">17</p> <table border="0" style="width: 100%; font-family: monospace;"> <thead> <tr> <th style="text-align: left;"></th> <th style="text-align: center;">↓</th> <th style="text-align: left;">Ala</th> <th style="text-align: left;">Val</th> <th style="text-align: left;">His</th> <th style="text-align: left;">Phe</th> <th style="text-align: left;">Asn</th> <th style="text-align: left;">Gly</th> <th style="text-align: left;">Gly</th> <th style="text-align: left;">Ala</th> <th style="text-align: left;">Glu</th> <th style="text-align: left;">Thr</th> <th style="text-align: left;">Arg</th> <th style="text-align: left;">His</th> <th style="text-align: left;">Phe</th> <th style="text-align: left;">Ser</th> <th style="text-align: left;">Val</th> <th style="text-align: left;">Tyr</th> <th style="text-align: left;">Glu</th> <th style="text-align: right;">↑</th> </tr> </thead> <tbody> <tr> <td style="padding-right: 10px;">Protein</td> <td></td> <td>C-</td><td>GU-</td><td>CAU</td><td>UUU</td><td>AAU</td><td>GG-</td><td>GG-</td><td>GC-</td><td>GAA</td><td>AC-</td><td>CG-</td><td>CAU</td><td>UUU</td><td>UC-</td><td>GU-</td><td>UAU</td><td>GAA</td> <td></td> </tr> <tr> <td style="padding-right: 10px;">m-RNA</td> <td style="font-size: small;">5'</td> <td></td><td></td><td style="text-align: center;">C</td><td style="text-align: center;">C</td><td style="text-align: center;">C</td><td></td><td></td><td></td><td style="text-align: center;">G</td><td></td><td style="text-align: center;">AGA</td><td style="text-align: center;">C</td><td style="text-align: center;">C</td><td style="text-align: center;">AGU</td><td></td><td style="text-align: center;">C</td><td style="text-align: center;">G</td> <td></td> </tr> <tr> <td></td> <td></td> <td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td style="text-align: center;">G</td><td></td><td></td><td></td><td></td><td></td><td></td> <td></td> </tr> <tr> <td style="padding-right: 10px;">Probe</td> <td style="font-size: small;">3'</td> <td>G-</td><td>CA-</td><td>GTA</td><td>AAA</td><td>TTA</td><td>CC-</td><td>CC-</td><td>CG-</td><td>CTT</td><td>TG-</td><td>GC-</td><td>GTA</td><td>AAA</td><td>AG-</td><td>CA-</td><td>ATA</td><td>CTT</td> <td></td> </tr> <tr> <td></td> <td></td> <td></td><td></td><td style="text-align: center;">G</td><td style="text-align: center;">G</td><td style="text-align: center;">G</td><td></td><td></td><td></td><td style="text-align: center;">C</td><td></td><td style="text-align: center;">TCT</td><td style="text-align: center;">G</td><td style="text-align: center;">GTCA</td><td></td><td style="text-align: center;">G</td><td style="text-align: center;">C</td> <td></td> </tr> <tr> <td></td> <td></td> <td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td style="text-align: center;">C</td><td></td><td></td><td></td><td></td><td></td><td></td> <td></td> </tr> </tbody> </table> </div>				↓	Ala	Val	His	Phe	Asn	Gly	Gly	Ala	Glu	Thr	Arg	His	Phe	Ser	Val	Tyr	Glu	↑	Protein		C-	GU-	CAU	UUU	AAU	GG-	GG-	GC-	GAA	AC-	CG-	CAU	UUU	UC-	GU-	UAU	GAA		m-RNA	5'			C	C	C				G		AGA	C	C	AGU		C	G														G								Probe	3'	G-	CA-	GTA	AAA	TTA	CC-	CC-	CG-	CTT	TG-	GC-	GTA	AAA	AG-	CA-	ATA	CTT						G	G	G				C		TCT	G	GTCA		G	C														C							
	↓	Ala	Val	His	Phe	Asn	Gly	Gly	Ala	Glu	Thr	Arg	His	Phe	Ser	Val	Tyr	Glu	↑																																																																																																																										
Protein		C-	GU-	CAU	UUU	AAU	GG-	GG-	GC-	GAA	AC-	CG-	CAU	UUU	UC-	GU-	UAU	GAA																																																																																																																											
m-RNA	5'			C	C	C				G		AGA	C	C	AGU		C	G																																																																																																																											
												G																																																																																																																																	
Probe	3'	G-	CA-	GTA	AAA	TTA	CC-	CC-	CG-	CTT	TG-	GC-	GTA	AAA	AG-	CA-	ATA	CTT																																																																																																																											
				G	G	G				C		TCT	G	GTCA		G	C																																																																																																																												
												C																																																																																																																																	
(57) Abstract																																																																																																																																													
<p>The present invention relates to genes and their encoded proteins which regulate neurite growth and the diagnostic and therapeutic use of such proteins (termed herein neurite growth regulatory factors). The proteins of the present invention include central nervous system myelin associated proteins and metalloproteases associated with glioblastoma cells and other malignant tumors which can metastasize to the brain. The CNS myelin associated proteins inhibit neurite outgrowth in nerve cells and neuroblastoma cells, and can also inhibit fibroblast spreading. Such inhibitory proteins include a 35,000 dalton and a 250,000 dalton molecular weight protein and analogs, derivatives, and fragments thereof. The CNS myelin associated inhibitory proteins may be used in the treatment of malignant tumors. The metalloproteases of the invention have value in the treatment of nerve damage and of degenerative disorders of the nervous system. The present invention is also directed to inhibitors of the metalloproteases. Such inhibitors in combination with the CNS myelin associated inhibitory proteins can be used in the treatment of malignant tumors.</p>																																																																																																																																													

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		
ES	Spain	MG	Madagascar		

NEURITE GROWTH REGULATORY FACTORS1. INTRODUCTION

5 The present invention is directed to genes and
their encoded proteins which regulate neurite growth,
antibodies thereto, and the therapeutic and diagnostic
uses of such proteins and antibodies. The proteins of
the present invention include central nervous system
10 myelin associated inhibitory proteins, and metallo-
proteases associated with malignant tumors, in
particular, primary brain tumors such as glioblastoma
and other tumors capable of metastasizing to and
spreading in the brain. The central nervous system
15 myelin associated inhibitory proteins inhibit neurite
outgrowth and fibroblast spreading and can have
important uses in the treatment of malignant tumors.
Antibodies to such inhibitory proteins can have uses
in the diagnosis of malignant tumors and in the
20 treatment of central nervous system damage and
degenerative nerve diseases. In a specific embodiment
of the invention, antibody to neurite growth inhibitor
may be used to promote the regeneration of neurons
over long distances following spinal cord damage. The
25 metalloproteases of the invention allow invasive
growth of glioblastomas and allow neurite outgrowth in
central nervous system tissue. They may have
important uses in the treatment of central nervous
system damage and degenerative nerve diseases.
30 Inhibition of the metalloprotease can be therapeutic-
ally useful in the treatment of malignant tumors.

2. BACKGROUND OF THE INVENTION

2.1. FACTORS INFLUENCING NEURITE GROWTH IN THE CENTRAL NERVOUS SYSTEM

5 Cell attachment, cell spreading, cell motility,
and, in particular, neurite outgrowth are strongly
dependent on cell-substrate interactions (Sanes, 1983,
Ann. Rev. Physiol. 45:581-600; Carbonetto et al.,
10 1987, J. Neurosci. 7:610-620). An increasing number
of substrate molecules favoring neuroblast migration
or neurite outgrowth have been found in central and
peripheral nervous tissue (Cornbrooks et al., 1983,
Proc. Natl. Acad. Sci. USA 80:3850-3854; Edelman,
1984, Exp. Cell Res. 161:1-16; Liesi, 1985, EMBO J.
15 4:1163-1170; Chiu, A.Y. et al., 1986, J. Cell Biol.
103:1383-1398; Fischer et al., 1986, J. Neurosci.
6:605-612; Lindner et al., 1986, Brain Res. 377:298-
304; Mirsky et al., 1986, J. Neurocytol. 15:799-815;
Stallcup et al., 1986, J. Neurosci. 5:1090-1101;
20 Carbonetto et al., 1987, J. Neurosci. 7:610-620). The
appearance of some of these factors can be correlated
with specific developmental stages, and, in the
peripheral nervous system (PNS), also with denervation
(Edelman, 1984, Exp. Cell Res. 161:1-16; Liesi, 1985,
25 EMBO J. 4:1163-1170; Stallcup et al., 1985, J.
Neurosci. 5:1090-1101; Daniloff et al., 1986, J. Cell
Biol. 103:929-945; Carbonetto et al., 1987, J.
Neurosci. 7:610-620). The extracellular matrix
protein tenascin has been shown to possess
30 nonpermissive substrate properties (Chiquet-Ehrismann
et al., 1986, Cell 47:131-139).

One of the most characterized of the soluble
factors favoring neurite outgrowth is nerve growth
factor (NGF). NGF promotes nerve fiber outgrowth from
35 embryonic sensory and sympathetic ganglia in vivo and
in vitro as well as neurite outgrowth (reviewed in

Thoenen et al., 1982, In: Repair and Regeneration of the Nervous System, J.G. Nicholls, ed., Springer-Verlag, NY, pp. 173-185). NGF may also guide the direction of such neurite outgrowth. Three different molecular forms of NGF have been recognized. One type is a dimer (molecular weight ~26,000) composed of two noncovalently linked, identical polypeptide chains. The second form is stable at neutral pH and contains three different polypeptide chains, α , β and γ (molecular weight ~140,000). The β chain is the biologically active chain and is identical to the first form of NGF. The third form, which is isolated primarily from mouse L cells, (see U.S. Patent No. 4,230,691, by Young, issued October 28, 1980, and references therein) has a molecular weight of about 160,000 but is unstable at neutral pH. NGF has thus far been isolated from the submandibular glands of mice, mouse L cells, and the prostate gland of the guinea pig and bull (reviewed in Thoenen et al., 1982, supra). No differences between the biological action of mouse, guinea pig and bull NGF have been detected. In addition, NGF isolated from mice have been found to bind to the human NGF receptor (Johnson et al., 1986, Cell 47:545-554).

The differentiated central nervous system (CNS) of higher vertebrates is capable of only very limited regenerative neurite growth after lesions. Limited regeneration after lesion has been seen in the retina (McConnell and Berry, 1982, Brain Res. 241:362-365) and in aminergic unmyelinated fiber tracts after chemical (Bjorklund and Stenevi, 1979, Physiol. Rev. 59:62-95) but not mechanical lesions (Bregman, 1987, Dev. Brain Res. 34:265-279).

Davis et al. (1987, Science 236:1101-1109) and Gage et al. (1988, Exp. Brain Res. 72:371-380)

observed a limited ingrowth of fibers into the hippocampus at 8-12 weeks. The longest growth of cholinergic fibers reinnervating the hippocampus was reported by Kromer et al. (1981, Brain Res. 210:153-200) and Tuszynski et al. (1990, Neurosci. 36:33-44), using embryonic tissue bridges. In these experiments, only short AchE-positive fibers were seen in the hippocampus at 4-6 weeks after operation. However, after very long survival times of 3-14 months, AchE-positive fibers were seen to extend over 2.5-3.5 mm. This very slow regeneration over longer distances was interpreted in the context of "glial reactions" in the denervated hippocampus (Kromer et al., 1981, Brain Res. 210:153-200; Gage et al., 1988, Exp. Brain Res. 72:371-380). Other studies have entailed direct grafting of cholinergic neurons into the denervated hippocampus (Bjorklund and Stenevi, 1984, Ann. Rev. Neurosci. 7:279-308; Gage et al., 1987, *in* Progr. Brain Res., Seil et al. (eds.), Amsterdam: Elsevier, pp. 335-347, for review). Electrophysiological evidence for the reformation of septo-hippocampal connections across bridges has also been obtained (Segal et al., 1981, Neurosci. Letters 27:7-12). The finding that grafting of other types of embryonic cholinergic neurons gives rise to an aberrant cholinergic innervation (Nilsson et al., 1988, J. Comp. Neurol. 268:204-222) shows a high level of specificity in guidance cues in the adult hippocampus.

Neurite growth from implanted embryonic CNS tissues in adult rat CNS has been found in some cases to reach up to 14 mm within some gray matter areas, but has not been found to exceed 1 mm within white matter (Nornes et al., 1983, Cell Tissue Res. 230:15-35; Bjorklund and Stenevi, 1979, Physiol. Rev. 59:62-95; Commission, 1984, Neuroscience 12:839-853). On

the other hand, extensive regenerative growth has been found in the CNS of lower vertebrates and in the peripheral nervous system of all vertebrates including
5 man.

Results from transplantation experiments indicate that the lack of regeneration is not an intrinsic property of CNS neurons, as these readily extend processes into implanted peripheral nervous tissue
10 (Benfey and Aguayo, 1982, Nature (London) 296:150-152; Richardson et al., 1984, J. Neurocytol. 13:165-182 and So and Aguayo, 1985, Brain Res. 328:349-354). PNS neurons, however, failed to extend processes into CNS tissue, thus indicating the existence of fundamental
15 differences between the two tissues (Aguayo et al., 1978, Neurosci. Lett. 9:97-104; Weinberg and Spencer, 1979, Brain Res. 162:273-279).

One major difference between PNS and CNS tissue is the differential distribution of the neurite
20 outgrowth promoting extracellular matrix component laminin (Liesi, 1985, EMBO J. 4:2505-2511; Carbonetto et al., 1987, J. Neurosci. 7:610-620). Other factors though may be involved. Drastic differences have been observed in neurite growth supporting properties of
25 sciatic and of optic nerve explants in vitro, in spite of the presence of laminin immunoreactivity in both explants (Schwab and Thoenen, 1985, J. Neurosci. 5:2415-2423). These experiments were carried out in the presence of optimal amounts of neurotrophic
30 factors and differences persisted upon freezing of tested substrates.

It has been suggested that the differentiated CNS may lack cellular or substrate constituents that are conducive for neurite growth during development
35 (Liesi, 1985, EMBO J. 4:2505-2511; and Carbonetto et al., 1987, J. Neurosci. 7:610-620), or it may contain

components which are nonpermissive or inhibitory for nerve fiber regeneration (Schwab and Thoenen, 1985, J. Neurosci. 5:2415-2423).

- 5 Recently, a growth (cell proliferation) inhibitory factor for mouse neuroblastoma cells was partially purified and characterized from the culture medium of fetal rat glioblasts as well as from C6 rat glioma cells (Sakazaki et al., 1983, Brain Res. 10 262:125-135). The factor was estimated to have a molecular weight of about 75,000 by gel filtration with BioGel P-20 with an isoelectric point of 5.8. The factor did not appear to alter the growth rate or morphology of glial cells (C6) or fibroblasts (3T3).
15 In addition, no significant nerve growth inhibitory factor activity was detected towards neuroblastoma cells (Neuro La, NS-20Y and NIE-115) or cloned fibroblasts (3T3).

20 2.2. PROTEASES AND THEIR INHIBITORS

- Different proteolytic activities have in the past been shown to be increased in tumorigenic cell lines (Matrisian et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:9413-9417; Mignatti et al., 1986, Cell 47:487-498),
25 in primary tumor explants (Mullins and Rohrllich, 1983, Biochem. Biophys. Acta 695:177-214), or in transformed cells (Quigley, 1976, J. Cell Biol. 71:472-486; Mahdavi and Hynes, 1979, Biochem. Biophys. Acta 583:167-178; Chen et al., 1984, J. Cell Biol. 98:1546-
30 1555; Wilhelm et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84: 6725-6729). One such group of proteases, metalloproteases has been shown to be involved in a number of membrane events, including myoblast fusion (Couch and Stritmatter, 1983, Cell 32:256-265), and
35 exocytosis in mast cells (Mundy and Stritmatter, 1985, Cell 40:645-656).

The isolation and characterization of a plasma membrane-bound metalloprotease (endopeptidase 24.11, enkephalinase) was reported by Almenoff and Orlowski
5 (1983, Biochemistry 22:590-599). A metalloprotease expressed by Rous sarcoma virus transformed chick embryo fibroblasts which degrades fibronectin and which was localized at adhesion sites and on "invadopodia" was described by Chen and Chen (1987,
10 Cell 48:193-203).

Studies indicate that proteases and their inhibitors can influence neurite extension in neuroblastoma cells (Monard et al., 1983, Prog. Brain Res. 58:359-363) and in cultured neonatal mouse
15 sensory ganglia (Hawkins and Seeds, 1986, Brain Res. 398:63-70). Cultured glial cells and gliomas were found to release a 43 kD protein, a glia derived neurite promoting factor (GdNPF), which induces neurite outgrowth in neuroblastoma cells but inhibits
20 cell migration (Monard, et al., 1983, supra). GdNPF was shown to be a very potent inhibitor of cell surface associated serine protease activity. Neurite outgrowth from normal mouse sensory ganglia can be enhanced by the addition of serine protease
25 inhibitors, ovomucoid trypsin inhibitor, leupeptin, soybean trypsin inhibitor, or thrombin (Hawkins and Seeds, 1986, supra). In contrast, proteases were found to inhibit such neurite outgrowth. Results from preliminary studies indicate that such proteases
30 possess a thrombin or trypsin like activity (Hawkins and Seeds, 1986, supra).

Other proteases have also been characterized though their functional role in neurite outgrowth is as yet unknown. These include a urokinase-like
35 plasminogen activator and a calcium dependent metalloprotease released by sympathetic and sensory

rat neurons (Pittman, 1985, Dev. Biol. 110:911-101).
The metalloprotease was found to have a molecular
weight of 62 kD, to require 1 mM Ca^{2+} for calcium
5 activity, and to degrade native and denatured collagen
more readily than casein, albumin, or fibronectin.
The plasminogen activator was found to have a
molecular weight of 51 kD, and was precipitated by a
rabbit antiserum produced against human urokinase. It
10 may be converted to its active form of 32 kD.

2.3. NEUROBLASTOMA

Neuroblastoma arises from neuroectoderm and
contains anaplastic sympathetic ganglion cells
15 (reviewed in Pinkel and Howarth, 1985, In: Medical
Oncology, Calabrese, P., Rosenberg, S.A., and Schein,
P.S., eds., MacMillan, NY, pp. 1226-1257). One
interesting aspect of neuroblastoma is that it has one
of the highest rates of spontaneous regression among
20 human tumors (Everson, 1964, Ann. NY Acad. Sci.
114:721-735) and a correlation exists between such
regression and maturation of benign ganglioneuroma
(Bolande, 1977, Am. J. Dis. Child. 122:12-14).
Neuroblastoma cells have been found to retain the
25 capacity for morphological maturation in culture. The
tumors may occur anywhere along the sympathetic chain,
with 50% of such tumors originating in the adrenal
medulla.

Neuroblastoma affects predominantly preschool
30 aged children and is the most common extracranial
solid tumor in childhood, constituting 6.5% of
pediatric neoplasms. One half are less than two years
of age upon diagnosis. Metastases are evident in 60%
of the patients at presentation usually involving the
35 bones, bone marrow, liver, or skin. The presenting
symptoms may be related to the primary tumor (spinal

coral compression, abdominal mass), metastatic tumor (bone pain) or metabolic effects of substances such as catecholamines or vasoactive polypeptides secreted by the tumor (e.g. hypertension, diarrhea).

Experimental evidence indicates that an altered response to NGF is associated with neuroblastoma (Sonnenfeld and Ishii, 1982, J. Neurosci. Res. 8:375-391). NGF stimulated neurite outgrowth in one-half of the neuroblastoma cell lines tested; the other half was insensitive. However, NGF neither reduced the growth rate nor enhanced survival in any neuroblastoma cell line.

Present therapies for neuroblastoma involve surgery and/or chemotherapy. Radiation therapy is used for incomplete tumor responses to chemotherapy. There is a 70-100% survival rate in individuals with localized tumors, but only a 20% survival rate in those with metastatic disease even with multiagent chemotherapy. It appears that patients less than one year have a better prognosis (70%) than older children.

2.4. GLIOBLASTOMA

Glioblastoma is a highly malignant astrocytic tumor usually located in the cerebral hemisphere. Astrocytes appear to be a supporting tissue for neurons and comprise the vast majority of the intraparenchymal cells of the brain (reviewed in Cutler, 1987, In: Scientific American Medicine V. 2, Rubenstein and Federman, eds., Scientific American, Inc., NY, pp. 1-7). Results from a survey conducted by the National Institute of Neurological and Communicative Disorders and Stroke indicated that the incidence of primary brain tumors in the United States is approximately eight per 100,000, in which 20% of

those tumors are glioblastomas. These tumors are generally found in individuals between 45 and 55 years of age. The tumors may also involve multiple lobes and may rupture into the ventricular system or extend across the corpus collosum to the opposite hemisphere. Due to the resulting increase in intracranial pressure, symptoms of tumor growth include headache, nausea and vomiting, mental status changes, and disturbances of consciousness. Due to their highly invasive properties, glioblastomas are associated with a poor prognosis. Chemotherapeutic agents or radiotherapies may be used. However, patients generally do not survive longer than two years even with these therapies.

3. SUMMARY OF THE INVENTION

The present invention relates to genes and their encoded proteins which regulate neurite growth and the diagnostic and therapeutic uses of such proteins. Such proteins are termed herein neurite growth regulatory factors. The neurite growth regulatory factors of the present invention include, in one embodiment, central nervous system myelin associated proteins which inhibit neurite outgrowth, and are termed herein neurite growth inhibitory factors. Another embodiment of the invention is directed to neurite growth regulatory factors which are metalloproteases associated with malignant tumors, in particular, those tumors metastatic to the brain. Such metalloproteases enable the malignant cells to overcome the inhibitory CNS environment and invade large areas of brain and spinal cord.

The CNS myelin associated proteins inhibit neurite outgrowth in nerve cells and neuroblastoma cells and also inhibit the spreading of fibroblasts

and melanoma cells. Such inhibitory proteins include but are not limited to 35,000 dalton and a 250,000 dalton molecular weight proteins and analogs, derivatives, and fragments thereof. The CNS myelin associated inhibitory proteins may be used in the treatment of patients with malignant tumors which include but are not limited to melanoma and nerve tissue tumors (e.g., neuroblastoma). The absence of the myelin associated inhibitory proteins can be diagnostic for the presence of a malignant tumor such as those metastatic to the brain (e.g., glioblastoma). The present invention also relates to antagonists of the CNS myelin associated inhibitory proteins, including, but not limited to, antibodies, i.e. antibodies IN-1 or IN-2. Such antibodies can be used to neutralize the neurite growth inhibitory factors for regenerative repair after trauma, degeneration, or inflammation. In a further specific embodiment, monoclonal antibody IN-1 may be used to promote regeneration of nerve fibers over long distances following spinal cord damage.

The present invention further relates to neurite growth regulatory factor receptors and fragments thereof as well as the nucleic acid sequences coding for such neurite growth regulatory factor receptors and fragments, and their therapeutic and diagnostic uses. Substances which function as either agonists or antagonists to neurite growth regulatory factor receptors are also envisioned and within the scope of the present invention.

The metalloproteases of the present invention can be found associated with malignant tumors, in particular, those capable of metastasizing to the brain. In a specific embodiment, the metalloprotease is associated with membranes of glioblastoma cells.

The metalloproteases, and analogs, derivatives, and fragments thereof can have value in the treatment of nerve damage resulting from trauma, stroke, degenerative disorders of the central nervous system, etc. In another embodiment of the invention, the metalloprotease may be used in combination with antibodies to the neurite growth inhibitory factors to treat nerve damage.

The present invention is also directed to inhibitors of and/or antibodies to the metalloproteases of the invention. Such inhibitors and/or antibodies can be used in the diagnosis and/or treatment of malignant tumors such as those which can metastasize to the brain, including but not limited to glioblastomas. Alternatively, the metalloprotease inhibitors, in combination with CNS myelin associated inhibitory protein or analogs, derivatives, or fragments thereof, may be used in the treatment and/or diagnosis of malignant tumors including but not limited to glioblastoma, neuroblastoma, and melanoma.

3.1. DEFINITIONS

As used herein, the following terms shall have the meanings indicated:

AChE:	acetylcholinesterase
BSA:	bovine serum albumin
cbz-tyr-tyr:	carbobenzoxy-tyrosine-tyrosine
30 cbz-gly-phe-NH ₂ :	carbobenzoxy-glycine-phenylalanine-amide
cbz-ala-phe-NH ₂ :	carbobenzoxy-alanine-phenylalanine-amide
cbz-phe-phe-NH ₂ :	carbobenzoxy-phenylalanine-phenylalanine-amide
35 cbz-gly-phe-phe-NH ₂ :	carbobenzoxy-glycine-phenylalanine-phenylalanine-amide

	cbz-phe-ala-phe-tyr-NH ₂ (SEQ ID NO:1):	carbobenzoxy-phenylalanine-alanine-phenylalanine-tyrosine-amide
5	CNS:	central nervous system
	CST:	Corticospinal tract
	DMEM:	Dulbecco's Modified Minimal Essential Media
10	EDTA:	ethylenediamine tetracetate
	EGTA:	ethylene glycol-bis-(b-aminoethyl ether) - N,N,N'-N'-tetracetate
	FCS:	fetal calf serum
	FITC:	fluorescein isothiocyanate
15	GdNPF:	glial-derived neurite promoting factor
	GFAP:	glial fibrillary acid protein
	HBO:	highly branched oligodendrocyte
20	Hepes:	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
	IN-1:	a monoclonal antibody against gel-purified 250 kD CNS myelin associated inhibitory protein
25	IN-2:	a monoclonal antibody against gel-purified 35 kD CNS myelin associated inhibitory protein
	J1:	a cell adhesion molecule of molecular weight 160-180 kD
30	kD:	kilodalton
	Mab:	monoclonal antibody
	MW:	molecular weight
	N-CAM:	neural cell adhesion molecule
	NGF:	nerve growth factor
35		

	neurite growth	CNS myelin associated 35 kD and
	regulatory factors:	250 kD inhibitory proteins, and a
		glioblastoma cell membrane
		associated metalloprotease
5	PBS:	phosphate buffered saline
	PLYS:	poly-D-lysine
	PMSF:	phenylmethylsulfonyl fluoride
	PNS:	peripheral nervous system
10	PORN:	polyornithine
	SCG:	superior cervical ganglion
	SDS-PAGE:	sodium dodecyl sulfate-
		polyacrylamide gel electrophoresis
15	Tris:	Tris (hydroxymethyl) aminomethane

4. DESCRIPTION OF THE FIGURES

Figure 1. Sensory axons in IN-1-injected nerve explants. Electron micrographs from representative experiments as described in Table I. (a) Electron micrograph of IN-1-injected optic nerve 1 mm from the proximal stump; an axon bundle growing in direct contact with the myelin is shown. (b) In the presence of IN-1, numerous axons grew 3 mm into the optic nerve explant. Bar, 0.5 μ m.

Figure 2. C6, but not 3T3 cells, infiltrate optic nerve explants. Phase-contrast microphotographs of 10 μ m frozen sections of rat optic (a,b) or sciatic (c,d) nerve explants, after 2 weeks incubation with C6 (a,c) or 3T3 (b,d) cells. Cells were added to one tip of the explants. Infiltrated cells can be seen after cresyl violet staining in both sciatic nerves (c,d) but only for C6 cells in the optic nerve (a). Arrows in (b) point to few 3T3 cells adjacent to blood vessels. Bar, 0.2 mm.

Figure 3. C6, but not 3T3 or B16 cells attach and spread on CNS white matter of rat cerebellar frozen sections. Phase contrast micrographs of rat cerebellar frozen sections (25 μ m) on which C6 (a,b), 3T3 (c,d) or B16 (e) cells were cultured for 2 days. A clear difference on white matter (wm) emerges for 3T3 and B16 cells compared to C6 cells. gl: granular layer, ml: molecular layer. Gray matter is composed of granular and molecular layer. Bar, 0.3 mm.

Figure 4. C6 cells overcome the inhibitory substrate property of CNS myelin. Spreading of C6 (a), 3T3 (b) and B16 (c) cells on PLYS or CNS myelin. Spreading is calculated as described in Section 6.1.3., infra using electron micrographs. 0% spreading: round cells; 100% spreading was taken as the average value at 300 minutes.

Figure 5. 1,10-phenanthroline inhibits C6 spreading specifically on CNS myelin. C6 cells were cultured for 3 hours on the indicated substrates in the presence of increasing doses of 1,10-phenanthroline. Spreading was inhibited by low doses exclusively on CNS myelin. 1,10-phenanthroline concentrations above 0.5 mM exert a general toxic effect on all substrates. Spreading was quantified as indicated in Figure 4.

Figure 6. Degradation of CNS inhibitory substrate by C6 plasma membranes is 1,10-phenanthroline sensitive. Spreading of 3T3 cells on CNS myelin was induced by pretreatment of myelin with C6 plasma membranes. 1,10-phenanthroline abolished this effect. Shown are phase contrast micrographs of 3T3 cells on polylysine (PLYs) (a), on CNS myelin (b), on CNS myelin pretreated with C6 plasma membranes, and on CNS myelin pretreated with C6 plasma membranes (C6-PM) in the presence of 1,10-phenanthroline.

Figure 7. C6 cell attachment and spreading on CNS white matter of rat cerebellar frozen section is impaired by metalloprotease blockers. Phase contrast micrographs of C6 cells on rat cerebellar frozen sections cultivated in the presence of either 0.1 mM cbz-ala-phe (a) or 0.1 mM cbz-tyr-tyr (b). Inhibition of attachment and spreading is particularly evident in the center of the white matter (asterisks), but is also visible in the main white matter branches (arrows).

Figure 8. C6 cell infiltration into CNS explants is impaired by cbz-tyr-tyr. Cells were added to one tip of optic nerve explants (chamber cultures) in the presence of the metalloprotease inhibitor cbz-tyr-tyr, or of the control peptide cbz-ala-phe. 14 day old cultures were quantified. Infiltrated cells were counted in the first 1.3 mm of the explants. Each column represents the number of infiltrated cells per 0.1 mm. Only the most central part of the explants was considered (0.25 mm). Values represent means \pm SEM of two sets of experiments for a total of 8 explants.

Figure 9. An extracellular metalloproteolytic activity of C6 cells degrades the substrate cbz-FAF[¹²⁵I]Y-NH₂ (SEQ ID NO:2) [cbz-phe-ala-phe-¹²⁵I-tyr-NH₂]. Autoradiograms of C6 cell culture supernatants analyzed on thin-layer chromatography showing multiple degradation products of the radio-iodinated peptide cbz-FAF[¹²⁵I]Y-NH₂. At 37°C (c), one single product showed clear o-phenanthroline dependency (asterisk). Incubation at 4°C (a) or 20°C (b) reduced mostly the formation of the o-phenanthroline-insensitive products (arrows). Analysis was performed 45 min upon addition of MEMα containing 0.2 μM cbz-FAF[¹²⁵I]Y-NH₂, 10 μM leupeptin, 1 mM PMSF, 10 μM pepstatin, 0.1 mM bestatin

(solid circles) and, where noted (open circles), 0.8 mM o-phenanthroline to the C6 cell cultures.

Figure 10. Cell fractionation experiments

- 5 localize a cbz-FAF[¹²⁵I]Y-NH₂ (SEQ ID NO:2) degradative activity to plasma membranes. Autoradiogram of thin layer chromatography of substrate incubated with plasma membranes (I). For quantification the incubation medium was extracted with chloroform. The
10 organic phase extracted completely unreacted substrate (II), whereas degradation products were retained in the aqueous phase (III). 0.8 mM phenanthroline completely blocked the degradation (IV-VI).

- Figure 11. The substrate cbz-FAF[¹²⁵I]Y-NH₂ (SEQ
15 ID NO:2) is degraded at one single site by a metallo-endoprotease. Autoradiogram of thin layer chromatography showing bestatin effect on Y-NH₂ formation. c., plasma membranes; best, bestatin; phen, o-phenanthroline; o, undegraded substrate.
20 Plasma membrane activity was measured as described for Figure 10.

- Figure 12. pH-optimum of plasma membrane associated metalloendoprotease. (a) Rate of cbz-FAF[¹²⁵I]Y-NH₂ (SEQ ID NO:2) hydrolysis was measured at
25 different pH values. Activity peaks in the weak acidic range. Pepstatin (b, 10 μM) sensitive activity was maximal at pH 4.0, whereas o-phenanthroline (c, 0.6 mM) sensitive activity peaked in the range 5.5-7.0. In (d), plasma membrane activity was measured
30 with pH 0.5 steps, to determine the pH optimum which was 5.5-6.0. The incubation media were 100 mM Na-acetate/acetate (circles), 100 mM MES/HCl (squares),

100 mM TRIS/HCl (diamonds), and contained 100 mM NaCl and 10 μ M pepstatin.

Figure 13. Solubilization of metalloproteolytic activity requires detergent. Total metalloproteolytic activity associated to C6 plasma membranes (control) was sedimented almost completely as tested by measuring pellet and supernatant separately (a). A similar result was obtained with 1 M NaCl treatment (b). On the other hand, 0.5% w/v CHAPS solubilized 85% total activity (c). C: Control; P: pellet; S: supernatant.

Figure 14. Schematic diagram of the hippocampus, showing the caudal (C) or lateral (L) directions of distances measured. B: extracellular matrix bridge; E: entry point of regenerating fibers into hippocampus; R: rostral hippocampus; CdH: caudal hippocampus.

Figure 15. Amino acid composition of the HPLC peak II derived from rat and bovine 35 kD neurite inhibitory factor (NI-35). Values represent the ratio of each amino acid to the amount of aspartic acid.

Figure 16. N-terminal amino acid sequence of rat NI-35 derived of HPLC peak II. The N-terminal amino acid sequence (SEQ ID NO:4) of NI-35 is shown, as well as a predicted mRNA sequence (SEQ ID NO:5) encoding the protein sequence, and the predicted sequence of a DNA (SEQ ID NO:6) complementary to the RNA which can be used as a hybridization probe for the cloning of NI-35.

Figure 17. Internal amino acid sequence of rat NI-35 derived of HPLC peak II. The amino acid sequence (SEQ ID NO:7) of an internal peptide of NI-35 derived from Endoproteinase-Lys C cleavage is shown. Also shown is a predicted mRNA sequence (SEQ ID NO:8) encoding the protein sequence, and the predicted sequence of a DNA (SEQ ID NO:9) complementary to the

RNA which can be used as a hybridization probe for the cloning of NI-35.

5 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to genes and their encoded proteins which regulate neurite growth and the diagnostic and therapeutic uses of such proteins. The proteins of the present invention
10 (termed herein neurite growth regulatory factors) include proteins associated with central nervous system myelin with highly nonpermissive substrate properties, termed herein neurite growth inhibitory factors. The neurite growth regulatory factors also
15 include metalloproteases which can be found associated with malignant tumors, in particular, those tumors metastatic to the brain.

The CNS myelin associated proteins of the invention inhibit neurite outgrowth in nerve cells or
20 neuroblastoma cells. The protein can also inhibit fibroblast spreading and migration. These inhibitory proteins are active cross-species and may be used in the treatment of patients with malignant tumors including but not limited to melanoma and tumors of
25 nerve tissue (e.g. neuroblastoma). In a specific example of the present invention, a 35 kilodalton and a 250 kilodalton CNS myelin associated protein are described. As demonstrated in a specific example of the invention, Section 8, infra, the 250 kD protein is
30 a complex containing the 35 kD protein.

The present invention is also directed to antibodies to and peptide fragments and derivatives of the neurite growth inhibitory proteins and their
therapeutic and diagnostic uses. These antibodies or
35 peptides can be used in the treatment of nerve damage resulting from, e.g., trauma (e.g., spinal cord

injuries), stroke, degenerative disorders of the central nervous system, etc. In particular, antibodies to CNS myelin associated proteins may be used to promote regeneration of nerve fibers. In a specific embodiment of the invention, monoclonal antibody IN-1 may be used to promote the regeneration of nerve fibers over long distances following spinal cord damage.

10 The present invention further relates to neurite growth regulatory factor receptors and peptide fragments thereof as well as the nucleic acid sequences coding for neurite growth regulatory factor receptors and fragments, and their therapeutic and
15 diagnostic uses. Antibodies to neurite growth regulatory factor receptors are also envisioned and within the scope of the present invention.

The present invention is also directed to metalloproteases associated with malignant tumors, in particular, those metastatic to the brain. In a specific embodiment, the metalloprotease is associated with glioblastoma cells. The metalloproteases of the invention are associated with the CNS infiltration activity of malignant cells, and can neutralize the inhibitory substrate properties of the CNS myelin-associated proteins. The metalloproteases can have therapeutic value in the treatment of nerve damage such as that resulting from traumatic injury (e.g. spinal cord injuries), stroke, degenerative disorders
25 of the central nervous system, etc. Alternatively, the metalloprotease may be used in combination with antibodies directed against myelin associated inhibitory proteins to treat nerve damage.

The present invention is also directed to
35 inhibitors of the metalloproteases. Such inhibitors can impair malignant cell spreading and infiltration,

and can be used in the treatment of malignant tumors (e.g. glioblastoma). In a specific embodiment, the metalloprotease inhibitors in combination with CNS
5 myelin associated inhibitory proteins such as the 35,000 dalton and/or the 250,000 dalton molecular weight proteins, may be used in the diagnosis and/or treatment of malignant tumors which include but are not limited to glioblastomas, neuroblastomas, and
10 melanomas.

The method of the invention can be divided into the following stages, solely for the purpose of description: (1) isolation and purification of neurite growth regulatory factors; (2) characteriza-
15 tion of neurite growth regulatory factors; (3) molecular cloning of genes or gene fragment encoding neurite growth regulatory factors; (4) production of antibodies against neurite growth regulatory factors; and (5) generation of neurite growth regulatory factor
20 related derivatives, analogs, and peptides. The method further encompasses the diagnostic and therapeutic uses of neurite growth regulatory factors and their antibodies.

25 5.1. ISOLATION AND PURIFICATION OF NEURITE GROWTH REGULATORY FACTORS

The present invention relates to CNS myelin associated inhibitory proteins of neurite growth, receptors of CNS myelin associated inhibitory proteins of neurite growth, and to metalloproteases such as
30 that associated with membranes of glioblastoma cells. The CNS myelin associated inhibitory proteins of the invention may be isolated by first isolating myelin and subsequent purification therefrom. Similarly, the metalloprotease may be obtained by isolation from
35 mammalian glioblastoma cells. Isolation procedures which may be employed are described more fully in the

sections which follow. Alternatively, the CNS myelin associated inhibitory proteins or the metalloprotease may be obtained from a recombinant expression system (see Section 5.3., infra). Procedures for the isolation and purification of receptors for the CNS myelin associated inhibitory proteins are described in Section 5.1.2., infra.

5.1.1. ISOLATION AND PURIFICATION OF CNS MYELIN ASSOCIATED INHIBITORY PROTEINS

CNS myelin associated inhibitory proteins can be isolated from the CNS myelin of higher vertebrates including, but not limited to, birds or mammals (both human and nonhuman such as bovine, rat, porcine, chick, etc.) (Caroni and Schwab, 1988, J. Cell Biol. 106:1281-1288). Myelin can be obtained from the optic nerve or from central nervous system tissue that includes but is not limited to spinal cords or brain stems. The tissue may be homogenized using procedures described in the art (Colman et al., 1982, J. Cell Biol. 95:598-608). The myelin fraction can be isolated subsequently also using procedures described (Colman et al., 1982, supra).

In one embodiment of the invention, the CNS myelin associated inhibitory proteins can be solubilized in detergent (e.g., Nonidet P-40™, sodium deoxycholate). The solubilized proteins can subsequently be purified by various procedures known in the art, including but not limited to chromatography (e.g., ion exchange, affinity, and sizing chromatography), centrifugation, electrophoretic procedures, differential solubility, or by any other standard technique for the purification of proteins (Caroni and Schwab, 1988, J. Cell Biol. 106:1281-1288). In one aspect, the solubilized proteins can be subjected to one dimensional electrophoresis, followed

by isoelectric focussing and elution from the focussing gel. Gel-eluted proteins can be acetone-precipitated, redissolved in 10% formic acid and
5 chromatographed on a C₄ reverse phase HPLC column (see Section 8, *infra*).

Alternatively, the CNS myelin associated inhibitory proteins may be isolated and purified using immunological procedures. For example, in one
10 embodiment of the invention, the proteins can first be solubilized using detergent (e.g., Nonidet P-40™, sodium deoxycholate). The proteins may then be isolated by immunoprecipitation with antibodies to the 35 kilodalton and/or the 250 kilodalton proteins.
15 Alternatively, the CNS myelin associated inhibitory proteins may be isolated using immunoaffinity chromatography in which the proteins are applied to an antibody column in solubilized form.

20 5.1.2. ISOLATION AND PURIFICATION OF
 RECEPTORS FOR THE CNS MYELIN
 ASSOCIATED INHIBITORY PROTEINS

Receptors for the CNS myelin associated inhibitory proteins can be isolated from cells whose attachment, spreading, growth and/or motility is
25 inhibited by the CNS myelin associated inhibitory proteins. Such cells include but are not limited to fibroblasts and neurons. In a preferred embodiment, neurons are used as the source for isolation and purification of the receptors.

30 In one embodiment, receptors to CNS myelin associated inhibitory proteins may be isolated by affinity chromatography of neuronal plasma membrane fractions, in which a myelin associated inhibitory protein or peptide fragment thereof is immobilized to
35 a solid support. Alternatively, receptor cDNA may be isolated by expression cloning using purified 35 kD or

250 kD neurite growth inhibitory factor as a ligand for the selection of receptor-expressing clones.

5 5.1.3. ISOLATION AND PURIFICATION OF
METALLOPROTEASES ASSOCIATED
WITH MALIGNANT TUMORS

The metalloproteases of the present invention may be isolated from cells of malignant tumors, in particular, glioblastomas. In a preferred embodiment, 10 a metalloprotease can be isolated from mammalian glioblastoma cells. In a preferred method, the metalloprotease is isolated from the plasma membrane fraction of such cells. The cells may be obtained by 15 dissociating and homogenizing tumors using procedures known in the art or from tumor cell lines. Plasma membrane fractions may be obtained using procedures known in the art, e.g., gradient centrifugation (Quigley, 1976, J. Cell Biol. 71:472-486). The 20 metalloprotease may be isolated from the membranes by solubilization with mild ionic or nonionic detergent (e.g., deoxycholate, Nonidet P-40™, Triton™, Brij™) using procedures described in the art (reviewed in Cooper, 1977, In Tools of Biochemistry, John Wiley & 25 Sons, NY, pp. 355-406) (see also Section 7, infra).

Purification of the metalloprotease can be carried out by known procedures, including but not limited to chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, electrophoretic procedures, differential 30 solubility, or by any other standard technique for the purification of proteins. Alternatively, an expression cloning system may be used by transfecting glioblastoma cDNA into, e.g., fibroblasts and 35 screening for a membrane associated metalloproteolytic activity.

5.2. PROTEIN CHARACTERIZATION

The neurite growth regulatory factors of the present invention can be analyzed by assays based on their physical, immunological, or functional properties. The half life of the neurite growth regulatory factors in cultured cells can be studied, for example, by use of cycloheximide, an inhibitor of protein synthesis (Vasquez, 1974, FEBS Lett. 40:563-584). In other experiments, a physiological receptor for a neurite growth regulatory factor could be identified by assays which detect complex formation with a neurite growth regulatory factor, e.g., by use of affinity chromatography with immobilized neurite growth regulatory factor, binding to a labeled neurite growth regulatory factor followed by cross-linking and immunoprecipitation, etc.

Electrophoretic techniques such as SDS-polyacrylamide gel electrophoresis and two-dimensional electrophoresis can be used to study protein structure. Other techniques which can be used include but are not limited to peptide mapping, isoelectric focusing, and chromatographic techniques.

The amino acid sequences of primary myelin associated inhibitors or of the metalloprotease can be derived by deduction from the DNA sequence if such is available, or alternatively, by direct sequencing of the protein, e.g., with an automated amino acid sequencer. The protein sequences can be further characterized by a hydrophilicity analysis (Hopp and Woods, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824-3828). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the protein (and the corresponding regions of the gene sequence, if available, which encode such regions).

Secondary structural analysis (Chou and Fasman, 1974, Biochemistry 13:222) can also be done, to identify regions of the CNS myelin associated inhibitor or glioblastoma metalloprotease sequence that assume specific secondary structures. Other methods of structural analysis can also be employed. These include but are not limited to X-ray crystallography (Engstrom, 1974, Biochem. Exp. Biol. 11:7-13) and computer modeling (Fletterick, R. and Zoller, M. (eds.), 1986, Computer Graphics and Molecular Modeling, in Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

15

5.3. MOLECULAR CLONING OF GENES
OR GENE FRAGMENTS ENCODING
NEURITE GROWTH REGULATORY FACTORS

5.3.1. ISOLATION AND CLONING OF THE NEURITE
GROWTH REGULATORY FACTOR GENES

Any mammalian cell can potentially serve as the nucleic acid source for the molecular cloning of the genes encoding the CNS myelin associated inhibitory proteins, including but not limited to the 35 kD and/or 250 kD myelin associated proteins (Caroni and Schwab, 1988 Neuron 1:85-96), or the glioblastoma associated metalloprotease, hereinafter referred to as neurite growth regulatory factor genes.

The DNA may be obtained by standard procedures known in the art from cloned DNA (e.g., a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired mammalian cell. (See, for example, Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd.,

Oxford, U.K., Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions, in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, a given neurite growth regulatory factor gene should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of a neurite growth regulatory factor gene from genomic DNA, DNA fragments are generated, some of which will encode the desired neurite growth regulatory factor gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNase in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment containing a neurite growth regulatory factor gene may be accomplished in a number of ways. For example, if an amount of a neurite growth regulatory factor gene or its specific RNA, or a fragment thereof, is available and can be purified and labeled, the generated DNA fragments may be screened by nucleic acid hybridization to the labeled probe (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961-3965). For example, in a preferred embodiment, a portion of a neurite growth regulatory factor amino acid sequence can be used to deduce the DNA sequence, which DNA sequence can then be synthesized as an oligonucleotide for use as a

hybridization probe. Alternatively, if a purified neurite growth regulatory factor probe is unavailable, nucleic acid fractions enriched in neurite growth regulatory factor may be used as a probe, as an initial selection procedure.

It is also possible to identify an appropriate neurite growth regulatory factor-encoding fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available. Further selection on the basis of the properties of the gene, or the physical, chemical, or immunological properties of its expressed product, as described supra, can be employed after the initial selection.

A neurite growth regulatory factor gene can also be identified by mRNA selection using nucleic acid hybridization followed by in vitro translation or translation in *Xenopus* oocytes. In an example of the latter procedure, oocytes are injected with total or size fractionated CNS mRNA populations, and the membrane-associated translation products are screened in a functional assay (3T3 cell spreading). Pread-sorption of the RNA with complementary DNA (cDNA) pools leading to the absence of expressed inhibitory factors indicates the presence of the desired cDNA. Reduction of pool size will finally lead to isolation of a single cDNA clone. In an alternative procedure, DNA fragments can be used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified neurite growth regulatory factor DNA, or DNA that has been enriched for neurite growth regulatory factor sequences. Immunoprecipitation analysis or functional assays of the in vitro translation products of the isolated mRNAs identifies the mRNA and, therefore, the cDNA

fragments that contain neurite growth regulatory factor sequences. An example of such a functional assay involves an assay for nonpermissiveness in which
5 the effect of the various translation products on the spreading of 3T3 cells on a polylysine coated tissue culture dish is observed (Caroni and Schwab, 1988 J. Cell Biol. 106:1281-1288). In addition, specific mRNAs may be selected by adsorption of polysomes
10 isolated from cells to immobilized antibodies specifically directed against a neurite growth regulatory factor protein. A radiolabeled neurite growth regulatory factor cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a
15 template. The radiolabeled mRNA or cDNA may then be used as a probe to identify the neurite growth regulatory factor DNA fragments from among other genomic DNA fragments.

Alternatives to isolating the neurite growth
20 regulatory factor genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the neurite growth regulatory factor gene. Other methods are possible and within
25 the scope of the invention.

The identified and isolated gene or cDNA can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not
30 limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives.
35 Recombinant molecules can be introduced into host

cells via transformation, transfection, infection, electroporation, etc.

5 In an alternative embodiment, the neurite growth regulatory factor gene may be identified and isolated after insertion into a suitable cloning vector, in a "shot gun" approach. Enrichment for a given neurite growth regulatory factor gene, for example, by size fractionation or subtraction of cDNA specific to low
10 neurite growth regulatory factor producers, can be done before insertion into the cloning vector. In another embodiment, DNA may be inserted into an expression vector system, and the recombinant expression vector containing a neurite growth
15 regulatory factor gene may then be detected by functional assays for the neurite growth regulatory factor protein.

The neurite growth regulatory factor gene is inserted into a cloning vector which can be used to
20 transform, transfect, or infect appropriate host cells so that many copies of the gene sequences are generated. This can be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the
25 complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA
30 termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and neurite growth regulatory factor gene may be modified by
35 homopolymeric tailing.

Identification of the cloned neurite growth regulatory factor gene can be accomplished in a number of ways based on the properties of the DNA itself, or
5 alternatively, on the physical, immunological, or functional properties of its encoded protein. For example, the DNA itself may be detected by plaque or colony nucleic acid hybridization to labeled probes (Benton, W. and Davis, R., 1977, Science 196:180;
10 Grunstein, M. and Hogness, D., 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Alternatively, the presence of a neurite growth regulatory factor gene may be detected by assays based on properties of its expressed product. For example, cDNA clones, or DNA
15 clones which hybrid-select the proper mRNAs, can be selected which produce a protein that inhibits in vitro neurite outgrowth. If an antibody to a neurite growth regulatory factor is available, a neurite growth regulatory factor protein may be identified by
20 binding of labeled antibody to the putatively neurite growth regulatory factor-synthesizing clones, in an ELISA (enzyme-linked immunosorbent assay)-type procedure.

In specific embodiments, transformation of host
25 cells with recombinant DNA molecules that incorporate an isolated neurite growth regulatory factor gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants,
30 isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

If the ultimate goal is to insert the gene into virus expression vectors such as vaccinia virus or
35 adenovirus, the recombinant DNA molecule that incorporates a neurite growth regulatory factor gene

can be modified so that the gene is flanked by virus sequences that allow for genetic recombination in cells infected with the virus so that the gene can be
5 inserted into the viral genome.

After the neurite growth regulatory factor DNA-containing clone has been identified, grown, and harvested, its DNA insert may be characterized as described in Section 5.3.4, infra. When the genetic
10 structure of a neurite growth regulatory factor gene is known, it is possible to manipulate the structure for optimal use in the present invention. For example, promoter DNA may be ligated 5' of a neurite growth regulatory factor coding sequence, in addition
15 to or replacement of the native promoter to provide for increased expression of the protein. Many manipulations are possible, and within the scope of the present invention.

20 5.3.2. EXPRESSION OF THE CLONED NEURITE GROWTH REGULATORY FACTOR GENES

The nucleotide sequence coding for a neurite growth regulatory factor protein or a portion thereof, can be inserted into an appropriate expression vector,
25 i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translation signals can also be supplied by the native neurite growth regulatory factor gene and/or its flanking regions. A variety of host-vector
30 systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); micro-
35 organisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid

DNA, or cosmid DNA. The expression elements of these vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one
5 of a number of suitable transcription and translation elements may be used.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric
10 gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination).

15 Expression vectors containing neurite growth regulatory factor gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the
20 first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to an inserted neurite growth regulatory factor gene. In the second
25 approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in
30 baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if a given neurite growth regulatory factor gene is inserted within the marker gene sequence of the vector, recombinants containing the neurite growth regulatory factor insert
35 can be identified by the absence of the marker gene function. In the third approach, recombinant

expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based on the physical, immunological, or functional properties of a given neurite growth regulatory factor gene product.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered neurite growth regulatory factor protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated

product. Expression in mammalian (e.g. COS) cells can be used to ensure "native" glycosylation of the heterologous neurite growth regulatory factor protein. Furthermore, different vector/host expression systems may effect processing reactions such as proteolytic cleavages to different extents.

5.3.3. IDENTIFICATION AND PURIFICATION OF THE EXPRESSED GENE PRODUCT

Once a recombinant which expresses a given neurite growth regulatory factor gene is identified, the gene product can be purified as described in Section 5.1, supra, and analyzed as described in Section 5.2, supra.

The amino acid sequence of a given neurite growth regulatory factor protein can be deduced from the nucleotide sequence of the cloned gene, allowing the protein, or a fragment thereof, to be synthesized by standard chemical methods known in the art (e.g., see Hunkapiller, et al., 1984, Nature 310:105-111).

In particular embodiments of the present invention, such neurite growth regulatory factor proteins, whether produced by recombinant DNA techniques or by chemical synthetic methods, include but are not limited to those containing altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine,

valine, proline, phenylalanine, tryptophan, and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, 5 asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are neurite 10 growth regulatory factor proteins which are differentially modified during or after translation, e.g., by glycosylation, proteolytic cleavage, etc.

15

20

25

30

35

PART II

5.3.4. CHARACTERIZATION OF THE NEURITE,
GROWTH REGULATORY FACTOR GENES

5 The structure of a given neurite growth regulatory factor gene can be analyzed by various methods known in the art.

The cloned DNA or cDNA corresponding to a given neurite growth regulatory factor gene can be analyzed by methods including but not limited to Southern
10 hybridization (Southern, 1975, J. Mol. Biol. 98:503-517), Northern hybridization (Alwine, et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5350-5354; Wahl, et al., 1987, Meth. Enzymol. 152:572-581), restriction
15 endonuclease mapping (Maniatis, et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), and DNA sequence analysis.

DNA sequence analysis can be performed by any techniques known in the art including but not limited
20 to the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-560), the Sanger dideoxy method (Sanger, et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5463-5467), or use of an automated DNA sequenator
25 (e.g., Applied Biosystems, Foster City, CA).

5.4. PRODUCTION OF ANTIBODIES TO NEURITE,
GROWTH REGULATORY FACTORS

Antibodies can be produced which recognize neurite growth regulatory factors or related proteins.
30 Such antibodies can be polyclonal or monoclonal.

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of a given neurite growth regulatory factor. For the production of antibody, various host animals
35 can be immunized by injection with a neurite growth regulatory factor protein, or a synthetic protein, or

fragment thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

A monoclonal antibody to an epitope of a neurite growth regulatory factor can be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256:495-497), and the more recent human B cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72) and EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In a particular embodiment, the hybridoma cell lines have been deposited with the European Collection of Animal Cell Cultures (ECACC). These cell lines have ECACC accession numbers 88102801 and 88102802. The production of these deposited hybridoma is detailed by Caroni and Schwab (1988, Neuron 1:85-96). These cell lines are used to express mouse monoclonal antibodies (IN-1 and IN-2) which recognize the 35 kD and 250 kD CNS myelin associated inhibitory proteins.

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous

techniques known in the art (e.g., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 5 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851, Takeda et al., 1985, Nature 314:452).

10 A molecular clone of an antibody to a neurite growth regulatory factor epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold 15 Spring Harbor, New York) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity 20 chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. 25 For example, such fragments include but are not limited to: the F(ab')₂ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragment, and the 30 2 Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

35 5.5. NEURITE GROWTH REGULATORY FACTOR-RELATED DERIVATIVES, ANALOGS, AND PEPTIDES

The production and use of derivatives, analogs, and peptid s related to a given neurite growth

regulatory factor are also envisioned, and within the scope of the present invention and include molecules antagonistic to neurite growth regulatory factors (for example, and not by way of limitation, anti-idiotypic antibodies). Such derivatives, analogs, or peptides which have the desired inhibitory activity can be used, for example, in the treatment of neuroblastoma (see Section 5.6, infra). Derivatives, analogs, or peptides related to a neurite growth regulatory factor can be tested for the desired activity by assays for nonpermissive substrate effects. An exemplary assay is the assay for nonpermissiveness. In this assay the effect of various translation products on the spreading of 3T3 cells on a polylysine coated tissue culture dish is observed (Caroni and Schwab, 1988, J. Cell Biol. 106:1281-1288).

The neurite growth regulatory factor-related derivatives, analogs, and peptides of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, a cloned neurite growth regulatory factor gene can be modified by any of numerous strategies known in the art (Maniatis, et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). A given neurite growth regulatory factor sequence can be cleaved at appropriate sites with restriction endonuclease(s), subjected to enzymatic modifications if desired, isolated, and ligated in vitro. In the production of a gene encoding a derivative, analogue, or peptide related to a neurite growth regulatory factor, care should be taken to ensure that the modified gene remains within the same translational reading frame as the neurite growth regulatory factor, uninterrupted by

translational stop signals, in the gene region where the desired neurite growth regulatory factor-specific activity is encoded.

5 Additionally, a given neurite growth regulatory factor gene can be mutated in vitro or in vivo, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endo-
10 nuclease sites or destroy preexisting ones, to facilitate further in vitro modification. Any technique for mutagenesis known in the art can be used, including but not limited to, in vitro site-directed mutagenesis (Hutchinson, et al., 1978, J.
15 Biol. Chem. 253:6551), use of TAB® linkers (Pharmacia), etc.

5.6. USES OF NEURITE GROWTH REGULATORY FACTORS

5.6.1. DIAGNOSTIC USES

20 5.6.1.1. CNS MYELIN ASSOCIATED INHIBITORY PROTEINS

CNS myelin associated inhibitory proteins, analogs, derivatives, and subsequences thereof, and anti-inhibitory protein antibodies or peptides have
25 uses in diagnostics. Such molecules can be used in assays such as immunoassays to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders affecting neurite growth extension, invasiveness, and regeneration. In one embodiment of
30 the invention, these molecules may be used for the diagnosis of malignancies. Alternatively, the CNS myelin associated inhibitory proteins, analogs, derivatives, and subsequences thereof and antibodies thereto may be used to monitor therapies for diseases
35 and conditions which ultimately result in nerve damage; such diseases and conditions include but are

not limited to CNS trauma, (e.g. spinal cord injuries), infarction, infection, malignancy, exposure to toxic agents, nutritional deficiency, paraneoplastic syndromes, and degenerative nerve diseases (including but not limited to Alzheimer's disease, Parkinson's disease, Huntington's Chorea, amyotrophic lateral sclerosis, progressive supra-nuclear palsy, and other dementias). In a specific embodiment, such molecules may be used to detect an increase in neurite outgrowth as an indicator of CNS fiber regeneration.

For example, in specific embodiments, the absence of the CNS myelin associated inhibitory proteins in a patient sample containing CNS myelin can be a diagnostic marker for the presence of a malignancy, including but not limited to glioblastoma, neuroblastoma, and melanoma, or a condition involving nerve growth, invasiveness, or regeneration in a patient. In a particular embodiment, the absence of the inhibitory proteins can be detected by means of an immunoassay in which the lack of any binding to anti-inhibitory protein antibodies (e.g., IN-1, IN-2) is observed.

The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, precipitation reactions, gel diffusion precipitation reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, immunoelectrophoresis assays, and immunohistochemistry on tissue sections, to name but a few.

In a specific embodiment, ligands which bind to a CNS myelin associated inhibitory protein can be used

in imaging techniques. For example, small peptides (e.g., inhibitory protein receptor fragments) which bind to the inhibitory proteins, and which are able to penetrate through the blood-brain barrier, when labeled appropriately, can be used for imaging techniques such as PET (positron emission tomography) diagnosis or scintigraphy detection, under conditions noninvasive to the patient.

Neurite growth inhibitory factor genes, DNA, cDNA, and RNA, and related nucleic acid sequences and subsequences, including complementary sequences, can also be used in hybridization assays. The neurite growth inhibitory factor nucleic acid sequences, or subsequences thereof comprising about at least 15 nucleotides, can be used as hybridization probes. Hybridization assays can be used to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with changes in neurite growth inhibitory factor expression as described supra. For example, total RNA in myelin, e.g., on biopsy tissue sections, from a patient can be assayed for the presence of neurite growth inhibitory factor mRNA, where the amount of neurite growth inhibitory factor mRNA is indicative of the level of inhibition of neurite outgrowth activity in a given patient.

5.6.1.2. CNS MYELIN ASSOCIATED INHIBITORY PROTEIN RECEPTORS

CNS myelin associated inhibitory protein receptors as well as analogs, derivatives, and subsequences thereof, and anti-receptor antibodies have uses in diagnostics. These molecules of the invention can be used in assays such as immunoassays or binding assays to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders affecting neurite growth, extension, invasion, and

regeneration. For example, it is possible that a lower level of expression of these receptors may be detected in various disorders associated with enhanced neurite sprouting and plasticity or regeneration such as those involving nerve damage, infarction, degenerative nerve diseases, or malignancies. The CNS myelin associated inhibitory protein receptors, analogs, derivatives, and subsequences thereof may also be used to monitor therapies for diseases and disorders which ultimately result in nerve damage, which include but are not limited to CNS trauma (e.g. spinal cord injuries), stroke, degenerative nerve diseases, and for malignancies.

The assays which can be used include but are not limited to those described supra in Section 5.6.1.1.

Neurite growth inhibitory factor receptor genes and related nucleic acid sequences and subsequences, including complementary sequences, can also be used in hybridization assays, to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with changes in neurite growth inhibitory factor receptor expression.

5.6.1.3. METALLOPROTEASES AND THEIR INHIBITORS

The metalloproteases of the invention, and their analogs, derivatives, and fragments thereof, as well as inhibitors and anti-metalloprotease antibodies, may be used for diagnostic purposes. These molecules of the invention may be used in assays such as immunoassays or inhibition type assays to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders affecting neurite growth extension, invasiveness, or regeneration. In a specific embodiment, the molecules of the present invention can be used to diagnose malignant tumors, in particular, glioblas-

toma, by detecting the presence of or an increase in metalloprotease levels. Alternatively, the molecules of the present invention may be used to monitor

5 therapies for malignant tumors such as glioblastoma by detecting changes in metalloprotease levels. In this latter embodiment, decreases or the disappearance of metalloprotease levels should can be indicative of treatment efficacy. In another embodiment, metallo-

10 protease levels can be relied upon as an indication of the malignant potential of a cell, e.g., a glial cell. "Malignant potential" as used herein shall mean those properties associated with malignant tumors, e.g. invasiveness, lethality, and/or metastatic potential.

15 While lack of metalloprotease activity in vitro does not necessary indicate a lack of malignant potential, the presence of such metalloprotease activity, and the level thereof, can be relied upon as an indication of the malignant potential of the cell expressing the

20 metalloprotease activity. In a preferred embodiment, metalloprotease activity is measured by a competitive substrate assay, e.g., using the peptide carbobenzoxy-Phe-Ala-Phe-Tyr-amide (SEQ ID NO:1) (cbz-FAFY-NH₂) as described in Section 7, infra.

25 The assays which can be used include but are not limited to those described supra in Section 5.6.1.1.

Metalloprotease genes and related nucleic acid sequences and subsequences, including complementary sequences, can also be used in hybridization assays,

30 to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with changes in metalloprotease expression as described supra. For example, total RNA in a sample (e.g., glial cells) from a patient can be assayed for the presence of

35 metalloprotease mRNA, where the presence or amount of metalloprotease mRNA is indicative of a malignancy in

the patient. In particular, a malignancy that can be metastatic to the brain (e.g., glioblastoma) can be detected.

5

5.6.2. THERAPEUTIC USES

5.6.2.1. CNS MYELIN ASSOCIATED INHIBITORY PROTEINS

CNS myelin associated inhibitory proteins of the present invention can be therapeutically useful in the treatment of patients with malignant tumors including, but not limited to melanoma or tumors of nerve tissue (e.g. neuroblastoma). In one embodiment, patients with neuroblastoma can be treated with the 35 kD and/or 250 kD proteins or analogs, derivatives, or subsequences thereof, and the human functional equivalents thereof, which are inhibitors of neurite extension. In another embodiment, a patient can be therapeutically administered both a CNS myelin-associated inhibitory protein and a metalloprotease inhibitor.

In an alternative embodiment, derivatives, analogs, or subsequences of CNS myelin inhibitory proteins which inhibit the native inhibitory protein function can be used in regimens where an increase in neurite extension, growth, or regeneration is desired, e.g., in patients with nervous system damage. Patients suffering from traumatic disorders (including but not limited to spinal cord injuries, spinal cord lesions, or other CNS pathway lesions), surgical nerve lesions, damage secondary to infarction, infection, exposure to toxic agents, malignancy, paraneoplastic syndromes, or patients with various types of degenerative disorders of the central nervous system (Cutler, 1987, In: Scientific American Medicines v. 2, Scientific American Inc., NY, pp. 11-1-11-13) can

be treated with such inhibitory protein antagonists. Examples of such disorders include but are not limited to Alzheimer's Disease, Parkinsons' Disease,
5 Huntington's Chorea, amyotrophic lateral sclerosis, progressive supranuclear palsy and other dementias. Such antagonists may be used to promote the regeneration of CNS pathways, fiber systems and tracts. Administration of antibodies directed to an epitope of
10 CNS myelin associated inhibitory proteins such as the 35 kD and/or 250 kD proteins, (or the binding portion thereof, or cells secreting such as antibodies) can also be used to inhibit 35 kD and/or 250 kD protein function in patients. In a particular embodiment of
15 the invention, antibodies directed to the 35 kD and/or 250 kD myelin associated inhibitory protein may be used to promote the regeneration of nerve fibers over long distances following spinal cord damage; in a specific example, monoclonal antibody IN-1 may be
20 used.

Various delivery systems are known and can be used for delivery of CNS myelin inhibitory proteins, related molecules, or antibodies thereto, e.g., encapsulation in liposomes or semipermeable membranes,
25 expression by bacteria, etc. Linkage to ligands such as antibodies can be used to target myelin associated protein-related molecules to therapeutically desirable sites in vivo. Methods of introduction include but are not limited to intradermal, intramuscular,
30 intraperitoneal, intravenous, subcutaneous, oral, and intranasal routes, and infusion into ventricles or a site of operation (e.g. for spinal cord lesions) or tumor removal. Likewise, cells secreting CNS myelin inhibitory protein antagonist activity, for example,
35 and not by way of limitation, hybridoma cells, encapsulated in a suitable biological membrane may be

implanted in a patient so as to provide a continuous source of anti-CNS myelin inhibiting protein antibodies.

5 In addition, any method which results in decreased synthesis of CNS myelin inhibitory proteins may be used to diminish their biological function. For example, and not by way of limitation, agents toxic to the cells which synthesize CNS myelin
10 inhibitory proteins (e.g. oligodendrocytes) may be used to decrease the concentration of inhibitory proteins to promote regeneration of neurons.

5.6.2.2. CNS MYELIN ASSOCIATED INHIBITORY PROTEIN RECEPTORS

15 CNS myelin associated inhibitory protein receptors or fragments thereof, and antibodies thereto, can be therapeutically useful in the treatment of patients with nervous system damage including but not limited to that resulting from CNS
20 trauma (e.g., spinal cord injuries), infarction, or degenerative disorders of the central nervous system which include but are not limited to Alzheimer's disease, Parkinson's disease, Huntington's Chorea, amyotrophic lateral sclerosis, or progressive
25 supranuclear palsy. For example, in one embodiment, CNS myelin associated inhibitory protein receptors, or subsequences or analogs thereof which contain the inhibitory protein binding site, can be administered to a patient to "compete out" binding of the inhibi-
30 tory proteins to their natural receptor, and to thus promote nerve growth or regeneration in the patient. In an alternative embodiment, antibodies to the inhibitory protein receptor (or the binding portion thereof or cells secreting antibodies binding to the
35 receptor) can be administered to a patient in order to prevent receptor function and thus promote nerve

growth or regeneration in the patient. Patients in whom such a therapy may be desired include but are not limited to those with nerve damage, stroke, or
5 degenerative disorders of the central nervous system as described supra.

Various delivery systems are known and can be used for delivery of CNS myelin associated inhibitory protein receptors, related molecules, or antibodies
10 thereto, e.g., encapsulation in liposomes, expression by bacteria, etc. Linkage to ligands such as antibodies can be used to target myelin associated protein receptor-related molecules to therapeutically desirable sites in vivo. Methods of introduction
15 include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, intranasal routes and infusion into ventricles or a site of tumor removal.

20 5.6.2.3. METALLOPROTEASES AND THEIR INHIBITORS

The metalloproteases of the present invention can be therapeutically useful in the treatment of various types of nervous system damage or degenerative disorders of the central nervous system (such as those
25 described supra, Section 5.6.2.2) In one embodiment, patients suffering from nervous system damage resulting from trauma, stroke, or neurodegenerative disorders can be treated with the metalloprotease or proteolytically active analogs, derivatives, or
30 subsequences thereof which stimulate neurite extension or regeneration of CNS fiber.

In an alternative embodiment, derivatives, analogs, or subsequences of the metalloproteases which antagonize or inhibit metalloprotease function, or
35 chemical inhibitors of the metalloprotease activity, can be used in regimens where an inhibition of

invasive migration and spread in the CNS is desired. Such inhibitors may include but are not limited to o-phenanthroline, EDTA, EGTA, cbz-tyr-tyr, cbz-gly-phe-NH₂, cbz-phe-phe-NH₂, and cbz-gly-phe-phe-NH₂. In a preferred embodiment, a competitive inhibitor such as carbobenzoxy-Phe-Ala-Phe-Tyr-amide (SEQ ID NO:1) (cbz-FAFY-NH₂) can be used. o-phenanthroline, EDTA, and EGTA may be obtained from commercial vendors (e.g. Sigma Chemical Co.). Cbz-tyr-tyr, cbz-gly-phe-NH₂, cbz-phe-phe-NH₂, and cbz-gly-phe-phe-NH₂ may also be obtained from commercial vendors (e.g. Vega Biotechnologies), or may be chemically synthesized. In specific embodiments, patients with various types of malignant tumors, in particular, those metastatic to the brain, can be treated with such inhibitors. In a preferred embodiment, a patient with a glioblastoma can be treated with such inhibitors. In another specific embodiment, administration of antibodies directed to an epitope of the metalloprotease can also be used to inhibit metalloprotease function in patients. In yet another specific embodiment of the invention, metalloprotease inhibitors and a CNS myelin associated inhibitory protein can both be administered to a patient for the treatment of a malignant tumor, examples of which include but are not limited to glioblastoma, neuroblastoma, or a melanoma.

Various delivery systems are known and can be used for the delivery of metalloproteases and related molecules, e.g., encapsulation in liposomes or semi-permeable membranes, expression by bacteria, etc. Linkage to ligands such as antibodies can be used to target molecules to therapeutically desirable sites in vivo. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, and intra-

nasal routes, and infusion into ventricles or a site of tumor removal.

5 6. INVOLVEMENT OF A METALLOPROTEASE IN
 GLIOBLASTOMA INFILTRATION INTO CENTRAL
 NERVOUS SYSTEM TISSUE IN VITRO

In the examples detailed herein, we describe a membrane-associated metalloprotease which plays a crucial role in the malignant tumor infiltration of
10 CNS tissue in vitro by the rat glioblastoma cell line C6.

We have discovered that malignant tumor infiltration of CNS tissue in vitro by the glioblastoma line C6, requires a plasma membrane bound
15 metallodependent degradative activity. C6 cells infiltrate optic nerve explants, attach and spread on white and grey matter of cerebellar frozen sections or on CNS myelin. The metal ions chelator 1,10-phenanthroline and the dipeptide cbz-tyr-tyr, but not
20 inhibitors for three other classes of proteases, blocked up to 67% of C6 cell spreading on CNS myelin. A metallodependent activity neutralizing CNS myelin inhibitory substrate properties toward 3T3 cells, is associated with a C6 plasma membrane fraction. The
25 same inhibitors of metalloprotease also impaired infiltration of CNS nerve explants and spreading on the CNS white matter of cerebellar frozen sections.

6.1. MATERIALS AND METHODS

30 6.1.1. CELL CULTURES

Rat C6, mouse NIH 3T3 and B16 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), usually to maximally 70-80% confluency. Cells were harvested
35 with a short trypsin treatment (0.1% in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's medium for 90 seconds) stopped by addition of

FCS in excess, collected by centrifugation. Cells were resuspended in either DMEM/FCS or defined serum-free medium (MEM) and used for experiments. Dissociated rat CNS glial cells were prepared starting from optic nerves of 6-7 days old Lewis rats as described below.

Optic nerves were dissected from 6-7 day old Wistar rats and collected in plating medium (air-buffered enriched L₁₅ with 5% rat serum; Mains and Patterson, 1973, J. Cell Biol. 59:329-345). The meninges and blood vessels were carefully removed under a microscope and the nerves were cut into small pieces. Dissociation of 10 day old nerves was done for 25 minutes twice in 0.25% trypsin (Sigma) and 0.02% collagenase (Worthington) (Raff et al., 1979, Brain Res. 174:283-318) in CMF-PBS (Ca⁺⁺/Mg⁺⁺ - free phosphate buffered saline) at 37°C. Adult optic nerves were dissociated in 0.1% trypsin, 0.1% collagenase for 1 hour at 37°C followed by 0.5% trypsin for 10 minutes. After washing and dissociation by trituration with a Pasteur pipet, the cells were plated into poly-D-lysine (PLYS) coated wells (100 mm², 100 µl medium) at a density of 20,000 cells per well. The culture medium was an enriched L15 medium with 5% rat serum, penicillin and streptomycin. C6, 3T3 and B16 cells were added to 2 day old cultures at a concentration of 30,000 cells per well, incubated for two hours and fixed with warm 4% formalin in phosphate buffer.

Inhibitory oligodendrocytes were identified by double labelling using the specific mouse monoclonal antibodies O₁ and O₄ (Sommer and Schachner, 1981, Dev. Biol. 83:311-327) as described below.

The specific antibodies were visualized by the corresponding anti-mouse, anti-rabbit or anti-goat -

fluorescein isothiocyanate (FITC) or - rhodamine isothiocyanate (RITC) linked secondary antibodies (Cappel, NC). Prior to staining, the cultures were
5 washed twice with PBS containing 5% sucrose and 0.1% bovine serum albumin (BSA). The antibodies were directed against surface antigens and were therefore incubated on the living cultures at room temperature for 30 minutes at a dilution of 1:20 in
10 PBS/sucrose/BSA.

For double labeling experiments with the O₄ antibody, living cultures were first incubated with antibody O₁ followed by anti-mouse-FITC, and then with antibody O₄ antigen; the sequence was reversed in some
15 experiments.

Double-labeled cultures were evaluated by systematically screening in the fluorescence microscope for the presence of one antigen (usually O₄), and every labeled cell was examined for the
20 presence of the other antigen, e.g. O₁.

6.1.2. PREPARATION OF NERVE EXPLANTS FOR INFILTRATION ASSAY

Optic nerve and sciatic nerve explants were
25 prepared as described (Schwab and Thoenen, 1985, J. Neurosci. 5:2415-2423). Briefly, the nerves were rapidly dissected from about 8 week old male rats, cleaned from the meninges, 3 times frozen and thawed using liquid nitrogen, and placed under a teflon ring (diameter 13 mm, thickness 1 mm) sealed to a culture
30 dish with silicon grease. Two chambers connected only by the explants were in this way obtained. 300,000 C6, 3T3 or B16 cells were plated in the inner chamber in DMEM/FCS and incubated for 5 to 20 days. The
35 medium was changed every other day. Cultures were fixed overnight with 4% formalin. The nerve xplants

were mounted with Tissue-Tek, 10 to 15 μ m sections were cut in a cryostat and collected on gelatine coated cover slips. After drying at room temperature overnight, the sections were stained in 0.75% cresyl violet, and evaluated. The infiltrated cells were counted for each 0.1 mm of the explants, starting from the tip where cells were added. Due to the 15 day incubation, the explants were often different in diameter. Therefore, only the central part of the nerves (0.25 mm) were considered, since only this part of the explants presented a good histological quality. Inhibition experiments were performed with nerve explants previously injected from both sides with 2 μ l of 3 mM cbz-tyr-tyr or cbz-ala-phe solutions.

6.1.3. CNS FROZEN SECTIONS AND MYELIN AS SUBSTRATES

Adult rat cerebellum frozen sections were prepared and dried on glass coverslips. 70,000 C6, 3T3, or B16 cells in 100 μ l were added to each well containing slices previously rinsed with cold DMEM/FCS. Cultures were incubated for 2 days at 37°C. Cultures were then fixed and stained with cresyl violet. Three to four cerebellum slices were used per point per experiment, with each experiment being repeated at least 2 times.

Myelin from rat spinal cord (CNS) or sciatic nerve (PNS) purified on a discontinuous sucrose gradient was prepared as described below.

Spinal cords were dissected from 200 g rats, carefully cleaned from adhering dorsal and ventral roots, and homogenized (polytron, 30 seconds at half maximal speed). Sciatic nerves were dissected, minced and homogenized. Myelin fractions were isolated by flotation of low speed supernatants on sucrose density gradients (Colman et al., 1982, J. Cell Biol. 95:598-

608). In some experiments, to remove possible trapped contaminants, the crude membrane fraction was washed following hypotonic shock. Sedimentation in hypotonic
5 medium was achieved at 10,000 x g for 5 minutes. Membrane fractions in sucrose solutions containing no more than 50 mM ionic species were adsorbed for several hours onto the wells of PLYS-coated tissue culture dishes (about 0.1 mg of protein per cm² of
10 tissue culture dish). Unbound membranes were removed by three washes with CMF-Hank's solution. Coated dishes were then immediately used in substrate testing experiments. In experiments with sympathetic or sensory neurons small droplets of central or
15 peripheral myelin were deposited in defined patterns over 35 mm culture dishes.

The myelin was then dried overnight onto PLYS coated wells (20 µg protein/well of 100 mm² surface). Unbound membranes were removed by three washes with
20 Ca²⁺/Mg²⁺-free Hank's solution. Myelin coated wells were immediately used in substrate testing assays by the addition of 9,000 cells (C6, 3T3, or B16) per cm². Alternatively, we used extracted CNS myelin protein, or SDS-PAGE purified 35 and 250 kD inhibitory proteins
25 reconstituted in liposomes (Caroni and Schwab, 1988, J. Cell Biol. 106:1281-1288). Experiments were scored at different time points using a phase contrast microscope equipped with a photcamera. Quantifications were done using a surface integration program; three
30 arbitrary fields were photographed for each well at a magnitude of 80X, at least 25 cells per picture were measured. Each point represents the mean of at least 3 wells ± SEM. Results are expressed as µ² of projected cell surface, or as degree, which was
35 calculated by subtracting from the projected surface

value of a spreading cell, the surface value of a completely spheric cell.

5 6.1.4. C6 PLASMA MEMBRANES AND CONDITIONED
 MEDIUM PREPARATION

C6 cells grown to 80% confluency were washed twice with Hank's medium, and harvested in 20 ml 8.5% sucrose, 50 mM NaCl, 10 mM Tris buffer, pH 7.4, using a rubber policeman. After mechanical homogenization
10 through a series of needles of decreasing size, a low purity plasma membrane fraction was obtained by centrifugation (5 minutes at 3000 x g, 10 minutes at 8000 x g, and then 2 hours at 100,000 x g). A higher purity fraction was isolated by loading the material
15 on a discontinuous sucrose gradient, containing 50 mM NaCl, 10 mM Tris, pH 7.4 (Quigley, 1976, J. Cell Biol. 71: 472-486). 20-40% sucrose interphase (C6 plasma membranes fraction) and 40-60% sucrose interphase (C6 mitochondrial fraction) were collected, washed in
20 Hank's medium and resuspended in MEM.

Conditioned media were obtained by cultivating 80% confluent C6 cell cultures for 1 day in MEM. The medium was then collected and centrifuged for 10 minutes at 3000 x g. In some experiments the
25 conditioned medium was concentrated 10 times using Centricon Tubes.

 6.1.5. TREATMENT OF CNS MYELIN
 WITH C6 PLASMA MEMBRANES

30 CNS myelin coated PLYS wells were prepared as described in the previous section, but instead of being immediately tested as substrate, they were first incubated with 50 µl of C6 plasma membranes (containing 0.8 mg protein/ml MEM) at 37° for 30 minutes.
35 Dishes were then rinsed twice with Hank's medium and immediately used as substrates for 3T3 cells. In some

experiments, protease blockers were added to the membranes using 10 times concentrated solutions.

5 6.2. RESULTS

6.2.1. C6 GLIOBLASTOMAS BUT NOT 3T3 FIBROBLASTS
 OR B16 MELANOMAS INFILTRATE OPTIC NERVE
 AND CNS WHITE MATTER IN VITRO

 Frozen optic nerve and sciatic nerve explants
were placed under a teflon ring and sealed with
10 silicon grease (Schwab and Thoenen, 1985, J. Neurosci.
5:2415-2423). C6 or 3T3 cells were plated into the
ring, in contact with one end of the nerve explants.
Culture medium was exchanged every other day, and
after 5 to 20 days of incubation the nerves were
15 fixed, and sectioned with a cryotome. Infiltrated
cells were recognized by cresyl violet staining. PNS
explants supported diffuse infiltration of both C6 and
3T3 cells (Figs. 2c, d). C6 cells were present in the
explants at higher density. In the optic nerve
20 explants, a different picture emerged (Figs. 2a, b);
3T3 cells did not infiltrate the nerves, with the
exception of very few cells which migrated along blood
vessels (Fig. 2b, arrow). On the other hand, C6 cells
infiltrated deep into the optic nerves with a diffuse
25 pattern, reaching a maximum distance of about 3 mm
from the entry point in 14 days (migration rate: about
0.2 mm/day).

 As an alternative model, adult rat cerebellum
frozen sections were used as a culture substrate for
30 C6, B16 or 3T3 cells. The highly metastatic B16
melanoma cells were found to clearly discriminate
between the substrate qualities of the grey and white
matter with regard to cell attachment, spreading and
migration. In fact, B16 cells exclusively attached
35 and spread on grey matter regions and, even if plated
at high cell densities, they did not attach on or

migrate into white matter areas of the sections (Fig. 3e). The same picture emerged for 3T3 cells, which formed dense monolayers on grey matter, but not on white matter (Figs. 3c, d). In contrast to B16 and 3T3 cells, C6 cells were found frequently on white matter as well as on grey matter (Fig. 3a, b). In some cases we found that C6 cells were more dense on the white matter than on the molecular layer of the grey matter, where they often formed little aggregates which spread with difficulty.

6.2.2. GLIOBLASTOMA CELL SPREADING IS NOT INHIBITED BY CNS MYELIN

The spreading behavior of C6 glioblastomas on CNS myelin adsorbed to PLYS coated wells was compared to that of B16 melanomas and 3T3 fibroblasts. B16 melanoma reaction to a CNS myelin substrate strongly resembled that of 3T3 fibroblasts: 3T3 or B16 cells spreading on CNS myelin was strongly impaired, whereas C6 cell spreading was slightly reduced at the beginning (90 minutes), but no further appreciable differences were detected at later time points (Fig. 4). The differences between cells on CNS myelin or on PLYS also persisted with prolonged incubation times (up to 1 day).

C6 cells were confronted with the SDS-PAGE purified inhibitors (35 kD and 250 kD) reconstituted in liposomes, and also with living, cultured oligodendrocytes. Again, 35 kD and 250 kD liposomes strongly inhibited 3T3 cell spreading, but they did not impair C6 cell spreading; C6 cells adhered and rapidly assumed the well spread characteristic "fried egg" appearance also on these reconstituted CNS myelin fractions.

6.2.3. SPECIFIC BLOCKERS OF METALLOPROTEASES INHIBIT C6 CELL SPREADING ON CNS MYELIN

The involvement of proteases in C6 behavior was investigated by determining the effect of inhibitors of proteases on C6 cell spreading on either CNS myelin or PLYS. Cys-, Ser- and Asp-protease blockers at the adequate concentrations had no discernible effect on C6 spreading on CNS myelin (Table I).

10

15

20

25

30

35

TABLE I
EFFECT OF DIFFERENT PROTEASE INHIBITORS ON C6
CELL SPREADING ON PLYS OR CNS MYELIN*

Protease Class	Protease Inhibitor	Spreading on:		Inhibition CNS (%)
		PLYS	% of Control on PLYS	
none				
control				
serine	6-amino-capronate	100	95	5
	hirudine	93	100	0
	PMSF	nq	nq	0
	trasyolol	100	94	6
	leupeptine	98	93	5
cystein	0.3 mM	91	83	8
aspartic	0.3 mM	98	95	3
metallo	1,10-phenanthroline	97	30	67
	bestatine	nq	104	0
	phosphoramidon	nq	91	9
	TIMP	102	93	9
	cm-phe-leu	95	92	3
	cbz-gly-gly-NH ₂	nq	99	1
	cbz-gly-phe-NH ₂	100	45	55
	cbz,ala-phe	98	90	8

TABLE I CONTINUED

	<u>Protease Class</u>	<u>Protease Inhibitor</u>	Spreading on:		Inhibition CNS (%)
			<u>PLYS</u>	<u>% of Control on PLYS</u>	
5	general	cbz-tyr-tyr	0.3 mM	101	56
		2-macroglobulin	30µM	70	52
		cocktail-		nq	nq
		cocktail+		nq	nq
10					45
					18
					0
					++

* Cells were plated on PLYS or CNS myelin coated culture dishes. Spreading was determined after 150 minutes as described supra in Materials and Methods. Inhibition values were calculated by subtracting spreading values on CNS myelin from the values on PLYS. Illustrated by Fig. 1.

PMSF: Phenyl methyl sulfonyl fluoride.

15 TIMP: Tissue inhibitor of metalloproteases.

Cocktail -: trasylol, 200 U/ml; leupeptine, 0.3 mM; pepstatine, 0.3 mM.

Cocktail +: same as cocktail -, but with 0.3 mM 1,10-phenanthroline.

nq: not quantified, only qualitative

Th specific metalloprotease blocker 1,10-phenanthroline on the other hand, resulted in a strong inhibition of C6 spreading specifically on CNS myelin: 1,10-phenanthroline inhibited C6 spreading on myelin up to 67% after 2 hours in culture (Table I). None of the blockers tested showed a significant effect on C6 cell spreading on PLYS. 1,10-phenanthroline is a general metalloprotease inhibitor due to its property of metal ion chelation. However, inhibition by this substance is not sufficient to define a proteolytic activity, since other metallodependent enzymes are also inhibited. Many other inhibitors of metalloproteases have been found, but they usually turned out not to be as general as 1,10-phenanthroline. Phosphoramidon (Komiyama, et al., 1975, Biochem. Biophys. Res. Comm. 65:352-357), bestatine (Umezawa, et al., 1976, J. Antibiot. 29:857-859) and the tissue inhibitor of metalloprotease (TIMP; Cawston, et al., 1987, Biochem. J. 195:159-165) did not impair C6 cell spreading (Table I).

TIMP also does not inhibit a brain membrane associated metalloprotease degrading enkephaline. Carboxymethyl-phe-leu (Fournie-Zaluski, M.C. et al., 1983, J. Med. Chem. 26:60-65), a modified peptide with high affinity for enkephalinase (Almenoff, J. and M. Orlowski, 1983, Biochemistry 22:590-599), did not inhibit C6 cell spreading (Table I). On the other hand, we found that the dipeptides cbz-gly-phe-NH₂ and cbz-tyr-tyr lead to 55% inhibition of C6 cell spreading on CNS myelin, but not on PLYS, PNS myelin or glass. These peptides are substrate peptides with metalloprotease specificity (Almenoff and Orlowski, supra; Baxter, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:4174-4178; Couch and Strittmatter, 1983, Cell 32:257-265; Chen and Chen, 1987, Cell 48:193-203;

Lelkes and Pollard, 1987, J. Biol. Chem. 262:15496-14505).

In order to exclude a possible general
5 enhancement of C6 cell spreading on nonpermissive
substrates, we tested metalloprotease-dependent C6
cell spreading on two other substrates in addition to
PLYS and CNS myelin (Fig. 5): PNS myelin and glass.
PNS myelin was chosen as a control for the general
10 properties of a myelin membrane fraction (e.g., high
content of lipids), and glass was chosen because of
its well known bad substrate qualities. Half maximal
inhibition of spreading on CNS myelin was obtained
with 200 μ M 1,10-phenanthroline. On PLYS, glass, and
15 PNS myelin (Fig. 5), 1,10-phenanthroline did not
impair C6 cell spreading at concentrations up to 0.5
mM (Fig. 5).

Absorption of CNS myelin with a monoclonal
antibody (IN-1) raised against CNS myelin inhibitory
20 components (Caroni and Schwab, 1988, Neuron 1:85-96),
largely reversed 1,10-phenanthroline dependent
inhibition of C6 cell spreading on CNS myelin
liposomes (Table II). IN-1 also almost completely
neutralized the inhibitory substrate property of CNS
25 myelin protein liposomes for 3T3 cells (Table II).

30

35

TABLE II

INHIBITION OF C6 CELL SPREADING BY
1,10-PHENANTHROLINE ON CNS MYELIN
IS NEUTRALIZED BY ANTIBODY IN-1*

Spreading value on:					
Cells	Antibody	1,10-Phenanthroline	CNS lipos	Plys	% inhibition on CSN lipos
3T3	-	0	1.11	2.00	45
3T3	IN-1	0	2.03	2.26	10
3T3	mouse IgM	0	1.16	2.18	47
C6	-	0	2.48	2.52	2
C6	-	0.3mM	1.35	2.49	46
C6	IN-1	0	2.46	2.48	1
C6	IN-1	0.3mM	2.25	2.54	11
C6	mouse IgM	0	2.36	2.42	2
C6	mouse IgM	0.3mM	1.41	2.39	41

20

*CNS myelin protein liposomes were used as substrates, and were preadsorbed with monoclonal antibody IN-1 against the myelin inhibitory substrate constituents (Caroni and Schwab, 1988, Neuron 1:85-96), or with mouse IgM. Spreading was calculated after 150 minutes and is expressed as $\mu\text{m}^2 \cdot 10^3$. % Inhibition relates to spreading values on PLYS.

25

These results indicate that the metalloprotease(s) plays an important role for overcoming of CNS myelin inhibitory substrates by neutralization of IN-1 inhibitory properties.

30

6.2.4. A C6 PLASMA MEMBRANE ASSOCIATED ACTIVITY NEUTRALIZES THE INHIBITORY SUBSTRATE PROPERTY OF CNS MYELIN

35

CNS myelin-coated culture wells were incubated with C6 conditioned medium or C6 plasma membranes, and

subsequently tested for their inhibitory substrate property with spreading of 3T3 cells. We found that C6 plasma membranes contained an activity which
 5 strongly reduced CNS myelin inhibitory activity (Fig. 6, Table III). The same treatment also decreased the inhibitory effect of CNS myelin protein liposomes or SDS-PAGE-purified, reconstituted 35 kD and 250 kD inhibitory components. The decrease in CNS myelin
 10 inhibitory activity for 3T3 cell adhesion and spreading was quantitized by measuring spreading values and DNA synthesis (Table III).

TABLE III

15 C6 PLASMA MEMBRANES REDUCE CNS MYELIN
INHIBITORY SUBSTRATE PROPERTY FOR 3T3 CELLS*

	Substrates	3T3 Cell Spreading (%)	³ H-Thymidine Incorporation
20	PLYS	100	100
	CNS myelin	15	30
	CNS myelin, C6 PM	52	83
	CNS myelin, C6 PM, phen. treated	17	50
	CNS myelin, C6 PM, EDTA treated	13	nd

25 *3T3 cells were plated on PLYS or CNS myelin. Spreading was assessed after 150 minutes CNS myelin was preincubated with a C6 cell plasma membrane fraction (C6 PM) in the absence or presence of metalloprotease inhibitors as indicated. ³H-thymidine was added when
 30 3T3 cells were plated, and incorporation was determined after 20 hours.

nd: not determined.

35 1,10-phenanthroline, EDTA, and the dipeptide cbz-gly-phe-NH₂ completely blocked the C6 plasma membrane effect. Trasylol, leupeptine and pepstatine did not

inhibit this effect. C6 conditioned medium used as such, or 10-times concentrated, did not contain any degradative activity able to naturalize CNS myelin
5 inhibitory substrate properties.

6.2.5. INHIBITORS OF METALLOPROTEASES IMPAIR
C6 CELL SPREADING ON CNS WHITE MATTER
AND C6 INFILTRATION OF CNS EXPLANTS

10 In order to investigate the relevance of the C6 plasma membrane metalloprotease activity not only for C6 cell attachment and spreading, but also for C6 cell migration and infiltration, C6 cells were plated on cerebellar frozen sections or added to optic nerve
15 explants in the presence of two metalloprotease inhibitors (1,10-phenanthroline and cbz-tyr-tyr). Parallel cultures contained inhibitors for the three other classes of proteases (leupeptine, pepstatine or trasylol), or a control dipeptide (cbz-ala-phe).

20 The presence of 1,10-phenanthroline at different concentrations (50, 100, 200 and 300 μ M), or the dipeptide cbz-tyr-tyr (100 μ M) dramatically changed the distribution and behavior of C6 cells on the white matter areas when cerebellar frozen sections were used
25 as culture substrates (Fig. 6). C6 cells also adhered in large numbers and spread extensively on the grey matter (Fig. 6).

Rat optic nerves were injected with 4 μ l of 3 mM solutions of either cbz-ala-phe or cbz-tyr-tyr. Cells
30 were incubated with medium containing 0.5 mM peptide. In the outer chamber, where no cells were present, the peptide concentration was 1 mM. After 14 days, the immigration of C6 cells into the explants differed greatly (Fig. 7). Cbz-ala-phe-injected nerves
35 contained more cells, and C6 cell infiltration was not affected, as compared to explants injected with culture medium only. On the other hand, cbz-tyr-tyr inhibited

C6 cell infiltration in all the 8 nerves examined (2 experiments). C6 cells were found mainly at the cut end of these nerve explants, and deep infiltration, which occurred massively in control explants, was strongly reduced by cbz-tyr-tyr.

6.3. DISCUSSION

The present results demonstrate that C6 glioblastoma cells, in contrast to neurons, fibroblasts and B16 melanoma cells, were not impaired in their migration into optic nerve explants or in attachment and spreading on CNS white matter, isolated CNS myelin, or living oligodendrocytes. The fact that the behavior of C6 cells differed characteristically from that of several cell types in all the assay systems studied suggests common underlying cell biological mechanisms, both for C6 spreading on an inhibitory substrate as well as for C6 mobility in an environment (optic nerve) which does not allow fibroblasts, Schwann cell or melanoma cell migration nor does it allow ingrowth of regenerating nerve fibers. This behavior of C6 cells was not due to "insensitivity" to the inhibitory components, since C6 cell motility was drastically inhibited on CNS myelin or white matter in the presence of specific protease blockers, and this effect was reversed by selective neutralization of myelin-associated inhibitory proteins with a monoclonal antibody (IN-1).

Inactivation of myelin-associated inhibitory constituents occurred by living C6 cells as well as by C6 plasma membranes. Our experiments with a number of protease blockers with different known specificities showed that this C6 associated activity belongs to the metalloenzyme family. The close parallelism observed between prevention of C6 cell spreading on CNS myelin

and prevention of inactivation of myelin-associated inhibitory proteins strongly suggests that modification of the inhibitory substrate components by a metallo-
5 protease could be the mechanism which enables C6 cells to spread on myelin, on white matter, and to infiltrate optic nerve explants.

Metalloproteases form an increasingly numerous group, the members of which differ in their sensitivity
10 to various blockers. The most general blocker is 1,10-phenanthroline which impaired C6 cell spreading on CNS myelin up to 67%, whereas most inhibitors of the other classes of proteases had no detectable effects. In the
15 early (90 minutes) but not the later (300 minutes) phases of C6 cell spreading on myelin, an effect of trypsin-like serine-protease inhibitors was also observed. The effect of 1,10-phenanthroline was dose-dependent, with an IC_{50} of 200 μM . This effect was
20 specific for CNS myelin as a substrate, since normal, rapid spreading of C6 cells was observed on other substrates such as CNS grey matter, PNS myelin, glass or PLYS in the presence of 1,10-phenanthroline. Other known metalloprotease blockers like bestatine
(inhibitor of aminopeptidases; Umezawa, et al., 1976,
25 J. Antibiot. 29:857-859), phosphoramidone (inhibitor of thermolysin-like metalloproteases; Komiyama, et al., 1975, Biochem. Biophys. Res. Commun. 65:352-357) and TIMP (inhibitor of ECM degrading metalloproteases; Cawston, et al., 1981, 195:159-165) did not lead to
30 inhibition of C6 cell spreading on CNS myelin. Since metalloproteases generally hydrolyze peptide bonds followed by large aliphatic or neutral aromatic amino acids, we tested the effect of dipeptide substrate analogues containing such residues. Cbz-gly-phe-NH₂ (1
35 mM) or cbz-tyr-tyr (0.3 mM) inhibited C6 cell spreading specifically on CNS myelin. Cbz-gly-phe-NH₂ was found

to inhibit other 1,10-phenanthroline sensitive enzyme activities with relative high specificity (Almenoff and Orlowski, 1983, Biochemistry 22:590-599; Baxter, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:4174-4178; 5 Couch and Strittmatter, 1983, Cell 32:257-265; Chen, J.M. and Chen, W.T., 1987, Cell 48:193-203; Lelkes and Pollard, 1987, J. Biol. Chem. 262:15496-14505).

Inactivity of C6-conditioned medium and cell 10 fractionation experiments demonstrated that the myelin-directed proteolytic activity is associated with C6 plasma membranes. The isolation and characterization of a plasma membrane-bound metalloprotease (endopeptidase 24.11, enkephalinase), which is also blocked by 15 1,10-phenanthroline but not by TIMP, was reported by Almenoff and Orlowski (1983, supra). However, the metalloprotease described herein is probably not an enkephalinase, since carboxymethyl-phe-leu, a peptide with high affinity for enkephalinase (Fournie-Zaluski, 20 et al., 1983, J. Med. Chem. 26:60-65), did not affect C6 spreading on myelin. A metalloprotease expressed by Rous sarcoma virus transformed chick embryo fibroblasts and localized at adhesion sites and on "invadopodia" was described by Chen, and Chen, 1987, supra. This 25 enzyme is also inhibited by 1,10-phenanthroline and cbz-gly-phe-NH₂, but not by phosphoramidon, as is the metalloprotease described here. However, unlike the enzyme of Chen and Chen, we could not detect any fibronectin degradative activity on C6 cells.

30 The highly metastatic B16 mouse melanoma cells were tested in all the assays used with C6 cells. Interestingly, B16 cells did not migrate into optic nerve explants, but responded to the myelin-associated inhibitors in a way very similar to 3T3 cells or 35 neurons. In line with this in vitro behavior, B16 cells, upon intraventricular injection, form mainly

meningiomas or intraventricular tumors without significant infiltration of the brain parenchyma. Thus, the mechanisms providing metastatic behavior to B16 cells in the periphery are different from those conferring high mobility to C6 cells in the CNS tissue.

Inhibition of C6-associated metalloprotease not only inhibited C6 spreading on CNS myelin, but also abolished C6 cell attachment, spreading, and migration on CNS white matter, and the dipeptide, cbz-tyr-tyr strongly impaired the migration of C6 cells into optic nerve explants. This metalloprotease activity(ies) may, therefore, be crucially involved in the infiltrative behavior of C6 glioblastoma cells in CNS tissue, also in vivo.

7. A PEPTIDE SUBSTRATE ASSAY FOR A METALLO-
ENDOPROTEASE INVOLVED IN C6 GLIOBLASTOMA
INFILTRATION INTO ADULT BRAIN WHITE MATTER

Using C6 glioblastoma cell spreading on CNS myelin as a model, we describe here a tetrapeptide, carbo-benzoxy-Phe-Ala-Phe-Tyr-amide (SEQ ID NO:1) (cbz-FAFY-NH₂), that acted as a competitive substrate to block C6 cell spreading on CNS myelin. The specific cleavage of this radio-iodinated substrate could be used as a sensitive, specific enzymatic assay to further characterize the metalloprotease involved in C6 invasive behavior in CNS tissue.

7.1. MATERIALS AND METHODS

Materials: Rat C6 glioblastoma cells were a kind gift of Prof. D. Monard (Friedrich Miescher Institute, Basel, Switzerland). Culture media were purchased from GIBCO BRL (Basel, Switzerland); cell culture plastic-ware were from Falcon (Becton Dickinson, Oxnard, CA). All chemicals were purchased from SIGMA (St. Louis, MO). Tri- and tetrapeptide protease blockers were

synthesized by BACHEM AG (Basel, Switzerland). 10^2 - 10^3 X stock solution of peptides, N-ethylmaleimide (NEM), iodacetamide and phenylmethanesulfonyl fluoride (PMSF) were prepared in dimethylsulfoxide (DMSO), while o-phenanthroline stock solution was prepared in ethanol. DMSO (0.5%) or ethanol (1%) were included in control incubations.

Carbobenzoxymethyl-L-glutamate-L-phenylalanine-L-phenylalanine-amide (cbz-GFF-NH₂) was synthesized from cbz-G-OH (I) and H₂N-FF-amide (II) as follows: 1 mmole of I was activated at -20°C with 1 mmole N-methylmorpholine and 1 mmole isobutylchloroformate in 10 ml tetrahydrofuran (THF). To start the reaction, 1 mmole of II in 5 ml THF was added. The reaction was continued by warming the reaction vessel to room temperature. The mixture was then stirred overnight. The product was crystallized by addition of a few drops of water, and was analysed by [¹H]NMR-spectrometry. The reaction profile was monitored on thin layer chromatography.

Cell Spreading Assay: Rat C6 glioblastoma cells (Benda et al., 1968, Science 161:370-371) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), usually to maximally 70-80% confluency (Paganetti et al., 1988, J. Cell Biol. 107:2281-2291). All cell culture media were routinely supplemented with penicillin and streptomycin. Cells were harvested by a short trypsin treatment (0.1% in Ca²⁺/Mg²⁺ -free [CMF] PBS/EDTA solution for 90 seconds) followed by addition of DMEM/10% FCS and centrifugation (600 X g, 6 min). The cell pellet was rinsed with 5 ml DMEM/10% FCS and centrifuged. For cell spreading assays, cells were resuspended at 10⁶/ml in defined serum-free medium (MEM α) supplemented with 0.2 μ g/ml insulin, 0.5 μ g/ml transferrin and 0.12 μ g/ml bovine serum albumin (BSA).

Protease blockers or peptides were added directly to the cell suspension from 10X solutions 10 min before plating.

- 5 Spinal cord myelin from young adult Lewis rats was purified on a discontinuous sucrose gradient (Colman et al., 1982, J. Cell Biol. 95:598-608; Schwab and Caroni, 1988, J. Neurosci. 8:2381-2393) and stored in 100 mM sucrose, 25 mM TRIS/HCl pH 7.4 at -80°C up to 6 months.
- 10 The myelin suspension (20 µg/100l) was incubated for 30 min on poly-D-lysine-coated wells (PLYS-wells). Unbound myelin membranes were removed by rinsing twice with CMF-Hank's and once with MEMα. Myelin-coated wells were used immediately for the spreading assay by
- 15 adding 9000 C6 cells per cm². Experiments were scored as described (Paganetti et al., 1988, J. Cell Biol. 107:2281-2291).

C6 Cell Fractionation and Preparation of

- Conditioned Medium: Preparation of plasma membrane from
- 20 C6 cells was performed according to (Raper and Kapfhammer, 1990, Neuron 2:21-29) with some modifications. Briefly, C6 cells were cultured in 30 culture dishes (13.5 cm diameter). At confluency, the culture medium was removed and the cell layers were frozen to -
- 25 20°C. All subsequent manipulations were performed at 4°C: cell debris were collected using a rubber policeman in 100 ml CMF-Hank's and further homogenized by 10 strokes in a Dounce homogenizer with teflon plunger. A small aliquot of this suspension was stored
- 30 at -20°C and used later (homogenate-fraction). The nuclear pellet was obtained by low speed centrifugation (10 min, 1000 X g) in a Sorvall S22 rotor and stored at -20°C. A crude vesicular fraction was obtained by centrifugating the nuclear supernatant in a Sorvall S22
- 35 rotor (20,000 X g, 30 min), and was resuspended in 10 ml CMF-Hank's. After adding 20 ml 2.25 M sucrose in

CMF-Hank's, this suspension was loaded on 4X 8 ml 2.25 M sucrose in CMF-Hank's and centrifuged at 80,000 X g for 1 h in a Beckman SW28 motor. Plasma membranes were harvested at the top and the mitochondrial fraction at the interphase of this 2 step gradient. Both fractions were diluted 10X with CMF-Hank's and pelleted (Beckman TI80, 22,000 X g, 30 min). The two pellets were resuspended in 2 ml MEM α (1.3 mg protein/ml) supplemented with 0.5 mM DTT and stored at (-20°C).

Conditioned medium was obtained as described (Paganetti et al., 1988, J. Cell Biol. 107:2281-2291) by collecting MEM α incubated for 1 day on a C6 cell layer.

Radio-iodination of cbz-FAFY-NH₂: Radio-iodination was performed using Iodo-Beads (PIERCE, IL, USA). Briefly, 400 μ l cbz-FAFY-NH₂ (SEQ ID NO:1) (200 μ g/ml) in 50 mM TRIS/HCl pH 7.3 and 1% sodium dodecylsulfate (SDS) were added to 100 μ l 0.5 M TRIS/HCl pH 7.3, 5 μ l ¹²⁵I Na (NEN, Germany, 100 μ Ci/l, 17.4 Ci/mg), and 3 Iodo-Beads. The reaction vessel was gently shaken every 1-2 min at room temperature for a total incubation time of 5 min. Radio-iodination was stopped by removing the Iodo-Beads. The probe was then diluted to 10 ml with 10% acetonitrile (ACN)/0.1% trifluor-acetic acid (TFA), loaded on a SEP-PAK cartridge (Waters/Millipore, MA, USA), rinsed with 10 ml 20% ACN/0.1% TFA and eluted with 1 ml 80% ACN, 0.1% TFA. This partially purified probe was then lyophilized, resuspended in 2 ml 10% ACN, 0.1% TFA (buffer A) and loaded on a reverse-phase column (300-5 C₁₈, Macherey-Nagel, Switzerland; HPLC System Gold, Beckman CA, USA). One main peak of radio-iodinated cbz-FAF[¹²⁵I]Y-NH₂ was eluted at 32 buffer B (80% ACN, 0.1% TFA). The specific activity of the probe was 10⁶ cpm/ μ g peptide (1 μ Ci/nM).

Enzyme Assay: For most experiments, 70-80% confluent cell layers (1 cm² wells, 100μl MEMα) or 2-5μg C6 plasma membrane (100μl MEMα) were used. Reactions were started by the addition of 10 μl cbz-FAF[¹²⁵I]Y-NH₂ (SEQ ID NO:2) (2 μM in MEMα, 10,000-30,000 cpm). If indicated, cell layers or plasma membrane suspensions were preincubated for 30 min with protease blockers or peptides. NEM or iodacetamide incubations of plasma membranes were performed at pH 8.5. The incubation was stopped by loading a 10 μl aliquot of the incubation medium on a thin layer chromatography plate (10 cm height, Kieselgel 60 F₂₅₄, MERCK, Germany) and air dried. Thin layer chromatography was routinely run with CHCl₃:CH₃OH:CH₃COOH 32% (5:3:1). Plates were then air dried and exposed on Kodak X-OMAT films (NY, USA) with an intensifying screen. To identify the degradation products on thin-layer chromatography, we run reference peptides under the same conditions.

For the quantification of the activity, the reaction was stopped by addition of an equal volume of chloroform. This simple procedure allowed us to separate an organic phase containing the unreacted substrate cbz-FAF[¹²⁵I]Y-NH₂ and an aqueous phase containing the charged degradation products (see also Fig. 10). Activity is expressed as % radioactivity contained in the aqueous phase. 100% is the sum of the radioactivity of both phases.

7.2. RESULTS

C6 glioblastoma cell spreading on CNS myelin-coated wells is impaired by short oligopeptides. C6 glioblastoma cells plated on CNS myelin as a culture substrate rapidly spread and overcome the inhibitory effect of the myelin-associated neurite growth inhibitors of 35 kD (NI-35) and 250 kD (NI-250) (most

other cell types remain round and are inhibited from spreading on this substrate). This behavior relies on a defined mechanism: the metalloprotease blockers o-phenanthroline, carbobenzoxy-Gly-Phe-amide (cbz-GF-NH₂) and cbz-YY specifically result in impaired cell spreading of C6 cells on NI-35/250 containing substrates (see supra; Paganetti et al., 1988, J. Cell Biol. 107:2281-2291). These blockers, due to their low metalloprotease affinity, were not suitable for the further biochemical characterization of the enzyme. Using C6 cell spreading on CNS myelin as an experimental model, we tested the effect of various other di-, tri- and tetra-peptides. C6 cells plated on CNS myelin formed large lamellipodia and were completely spread in less than 2 h. No differences were observed between cells plated on CNS myelin- or on control, PLYS-coated wells. In contrast, o-phenanthroline or cbz-GF-NH₂ impaired C6 cell spreading specifically on CNS myelin: the majority of cells spread poorly, and some did not spread at all (no effect of these compounds is seen on PLYS). The tripeptides cbz-GFF-OCH₃ and cbz-AFY-NH₂ had similar effects as o-phenanthroline, leading to strong inhibition of cell spreading. The tetrapeptide cbz-FAFY-NH₂ (SEQ ID NO:1) was the longest peptide tested that clearly impaired C6 cell spreading on CNS myelin. Control experiments were performed with peptides containing unrelated amino acid sequences (e.g. cbz-GGG-NH₂) or with partially unprotected peptides (e.g. AFY-NH₂). cbz-FAFY-NH₂ had no effect on PLYS-coated wells.

In Table IV, half-maximal inhibition values (IC₅₀) for each peptide tested are given.

TABLE IV

PEPTIDE CONCENTRATIONS RESULTING IN HALF
MAXIMAL INHIBITION OF C6 CELL SPREADING
(IC₅₀) on PLYS or CNS MYELIN *

	Compound	IC ₅₀ on PLYS	IC ₅₀ on CNS myelin
		[μ M]	[μ M]
10	1,10-phenanthroline	600	200
	cbz-GF-NH ₂	2000	600
	cbz-AF-NH ₂		600
	cbz-FA-NH ₂		600
	cbz-YY		300
15	cbz-GFF-NH ₂	100	8
	cbz-GFF		300
	cbz-AFY-NH ₂	300	20
	AFY-NH ₂		1000
20	cbz-FAFY-NH ₂ (SEQ ID NO:1)		3
	FAFY-NH ₂ (SEQ ID NO:3)		300

*Cells were plated on PLYS- or CNS myelin-coated culture dishes in the presence of the indicated compounds.
25 Spreading was determined after 2 h as described in Materials and Methods. Cell spreading inhibition values were calculated on the basis of increasing dilution of the compounds (each point corresponded to a factor-3 dilution). Each value is the mean of at least two experiments. SEM < 10%.

30 The dipeptides cbz-GF-NH₂ and cbz-AF-NH₂ required a concentration of 600 μ M for half-maximal inhibition of spreading on CNS myelin, whereas on PLYS concentrations up to 2 mM (saturation limit!) had no effect. The tripeptides
35 cbz-GFF-OCH₃ and cbz-GLF-NH₂ impaired C6 cell spreading on CNS myelin with significantly lower IC₅₀ (5 μ M). The most

potent blocker of C6 cell spreading on CNS myelin was the tetrapeptide cbz-FAFY-NH₂ (SEQ ID NO:1) with an half-maximal inhibition at 3 μ M. Protection of both amino- and carboxy-
5 terminals, seems to be a prerequisite for activity, since unprotected, charged peptides had no effect. This property suggests the involvement of an endoprotease rather than of an exopeptidase.

Cbz-FAFY-NH₂ is a competitive substrate. As
10 previously described, the tetrapeptide cbz-FAFY-NH₂ (SEQ ID NO:1) was the most potent blocker of C6 cell spreading on CNS myelin. As to its mechanism of action, cbz-FAFY-NH₂ could be acting either as an active-site ligand or as a competitive substrate. To obtain insight into the
15 inhibition mechanism, we labeled cbz-FAFY-NH₂ by radioiodination and incubated it on living cultures of C6 cells. In order to eliminate unspecific hydrolysis, we included blockers of the other three protease classes, which did not affect C6 cell spreading on CNS myelin. Cbz-FAF[¹²⁵I]Y-NH₂
20 (SEQ ID NO:2) (0.2 μ M) was readily degraded by living cells to multiple degradation products as shown by thin-layer chromatography (Fig. 9). Although various degradation products were detected under these conditions, only one of the products showed strong o-phenanthroline sensitivity
25 (Fig. 9a, asterisk). Incubation of cells at 4°C or at 20°C strongly reduced endocytosis; we found that at both these temperatures only o-phenanthroline insensitive activities were largely suppressed (Fig. 9b, c, arrows), whereas under these conditions the o-phenanthroline sensitive products
30 were still easily detected.

This result suggested that the tetrapeptide cbz-FAFY-NH₂ acted as a competitive substrate for the myelin protein degrading C6-associated protease.

Cbz-FAFY-NH₂ is degraded by a plasma membrane
35 associated metalloprotease. To define the specific cellular localization of the metalloproteolytic activity of C6 cells,

we assayed cbz-FAF[¹²⁵I]Y-NH₂ degradation by different subcellular fractions. Maximal activity was associated with the plasma membrane (2.7 nMol/min). On the other hand, crude homogenate (0.08 nMol/min) and mitochondrial fraction (0.27 nMol/min) were clearly less active. No activity was found in C6 cell conditioned medium (0.03 nMol/min (Fig. 10a). (All measurements were performed with the same amount (5 µg) of different cellular fractions).

Quantification of activity was achieved by extracting from the incubation medium the unreacted cbz-FAF[¹²⁵I]Y-NH₂ (SEQ ID NO:2) with chloroform; the degradation products remained in the aqueous phase due to their electrical charge (Fig. 10b). By measuring the radioactivity contained in either phases, it was possible to precisely determine the rate of degradation.

Characterization of a C6 plasma membrane associated metalloprotease. Two degradation products were detected upon incubation of cbz-FAF[¹²⁵I]Y-NH₂ with plasma membranes (see above). To identify these products, and the specific cleavage sites, we ran reference peptides containing the tyrosine-residue (see Materials and Methods). This procedure lead to the identification of two distinct radioiodinated products, FY-NH₂ and Y-NH₂, resulting from the hydrolyzation of cbz-FAF[¹²⁵I]Y-NH₂ (SEQ ID NO:2) by C6 plasma membranes. This suggested the involvement of at least two different proteolytic activities. Protease blocker studies were done to identify their specific subclass. Serine- (aprotinin, soybean trypsin inhibitor, PMSF), cysteine- (leupeptin, N-ethylmaleimide, iodoacetamide) and aspartyl-protease (pepstatin) blockers had no effect. In contrast to this, o-phenanthroline blocked completely either activities with an IC₅₀ of 0.2 mM (not shown). Since only metalloproteases can be involved, we have tested various metalloprotease specific blockers. Captopril (for angiotensin converting enzyme), phosphoramidon (for

collagenase and enkephalinase) and cm-LF (for enkephalinase) had no effect.

On the other hand, bestatin did not affect overall activity, but specifically inhibited the formation of Y-NH₂ (Fig. 11). Since bestatin is a well known aminopeptidase blocker, it could act only upon prior cleavage of cbz-FAFY-NH₂ (SEQ ID NO:1) to cbz-FA + FY-NH₂. This initial cleavage occurred by a metalloendoprotease insensitive to bestatin. From this we conclude that the activity in C6 plasma membranes cleaved the substrate cbz-FAFY-NH₂ (SEQ ID NO:1) at one single site, causing the formation of cbz-FA and FY-NH₂.

Subsequently, to obtain information on the specificity of the cleavage of cbz-FAFY-NH₂ (SEQ ID NO:1) by the C6 plasma membrane associated metalloendoprotease, we have tested several other known endoproteases.

TABLE V

DEFINED PROTEASES DEGRADE THE SUBSTRATE cbz-FAF[¹²⁵ I]Y-NH ₂ WITH DISTINCT AFFINITIES*			
Class	Protease	Amount necessary to degrade 50% of substrate in 30 min [μg]	
serine	trypsin	0.3	
	urokinase (uPA)	>100	
	tPA	>100	
cysteine	papain	2.0	
acidic	cathepsin D	>100 (pH 7.4)	
		0.005 (pH 4.0)	
metallo	collagenase	3.0	
	total C6 PM protein	1.5	

* The substrate cbz-FAF[¹²⁵I]Y-NH₂ (SEQ ID NO:2) was incubated initially with 100 μg for each protease. The amount of protease was then reduced stepwise by a factor of 3 for each further point. Activity was measured after 30 min (37°C, pH

7.4). Shown are the amounts of protease necessary to obtain degradation of 50% of the substrate.

0.3 μ g trypsin, 2 μ g papain and 3 μ g collagenase were
5 necessary to cleave 50% of cbz-FAFY-NH₂ (SEQ ID NO:1) in 30
min, 37°C (pH 7.4). Up to 100 μ g urokinase (uPA) and
tissue-type plasminogen activator (tPA) did not cleave cbz-
FAFY-NH₂ (SEQ ID NO:1). Half maximal activity for Cathepsin
D was obtained with 5 ng at pH 4.0, but no activity was
10 detected at pH 7.4 with up to 100 μ g protein. 1.5 μ g C6
plasma membranes resulted in 50% substrate cleavage (Table
V). Total degradation of the substrate by plasma membranes
was completed after 2h (not shown).

The rate of C6 metalloendoprotease activity was
15 measured at different pH values. We found activity in the
pH range of 4.0-8.5 (Fig. 12a). This broad range could be
due to the presence of a contamination of acidic proteases
in the plasma membrane fraction. To test for this, we
studied the effect of the general acidic protease blocker
20 pepstatin and of o-phenanthroline at the different pH
values. In fact, a pepstatin sensitive activity was found
at pH 4.0, but was absent at neutral pH (Fig. 12b). In
contrast, the o-phenanthroline sensitive activity was
maximal in the pH range of 5.5-7.0 (Fig. 12c), with peak
25 activity between pH 5.5 and 6.0 (Fig. 12d). This result
suggests that at low pH values total plasma membrane
activity was the sum of an aspartyl- and of a
metalloproteolytic activity. At pH 7.0, we were able to
measure only metalloproteolytic activity.

30 Solubilization of the activity. In order to
dissociate peripheral membrane proteins, C6 plasma membranes
were treated either with pH 11.0 or with high ionic strength
(1 M NaCl). Upon centrifugation, pellet and supernatant
were tested separately for protease activity using the
35 substrate cbz-FAF[¹²⁵I]Y-NH₂ (SEQ ID NO:2). Activity was
routinely measured at its maximum (30 min) and at pH 7.0.

Neither treatment extracted activity from the membrane fraction (Fig. 13a), suggesting the presence of a tightly associated or integral membrane enzyme. The presence of 1 M NaCl in the incubation buffer lead to an 87% increase of total metalloendoproteolytic activity (Fig. 13b). A similar result was obtained also with 2 M NaCl (65%) or 1.7 M ammonium sulfate (90%).

The detergent CHAPS (0.5% w/v) solubilized 85% of the total activity from plasma membranes (Fig. 13c). Extraction with this detergent also lead to a net increase in total enzyme activity (Fig. 13c). Maximal recovery of activity was found at a concentration of 0.5% w/v CHAPS. Under these conditions, a 4.4 fold increase of total activity was measured.

8. PURIFICATION AND N-TERMINAL SEQUENCE OF BOVINE AND RAT 35 KD INHIBITORY PROTEIN

1. Inhibitory activity was tested from bovine and rat crude CNS myelin protein extract, using the fibroblast spreading assay. Both species showed activity.

2. Gel eluted 35 kD and 250 kD proteins from bovine and rat CNS myelin, incorporated into liposomes, showed inhibitory activity that could be neutralized by IN-1 antibody.

3. Gel eluted (after 1-dimensional polyacrylamide gel electrophoresis) 33 kD, 35 kD and 250 kD proteins from bovine or rat CNS myelin sources were acetone precipitated, redissolved in 10% formic acid in H₂O and chromatographed on a C₄ reverse phase HPLC column. Proteins were chromatographed by gradients from 0.1% trifluoroacetic acid (TFA) in water to 0.1% TFA in 80:20 acetonitrile: water. The eluate absorbance was monitored at both 215 nm and 280 nm. HPLC fractions containing protein peaks were lyophilized, resuspended in sample buffer and subjected to ran on a SDS-PAGE under reducing conditions.

The HPLC chromatographs from bovine 33 kd, 35 kd and 250 kd gel eluted proteins show two common identical peaks, which elute at ~62% (peak I) and ~73% (peak II) acetonitrile. Aliquots of these peak fractions migrate at 33 kd on a SDS-PAGE gel. Interestingly, the amino acid compositions (Fig. 15) (determined by the orthophthaldialdehyde method) of peaks I and II derived from 33 kd or 35 kd or 250 kd material are very similar, which implies that they represent two subforms of one heterogenous protein. Preliminary N-terminal sequence data of peak II derived from 33 kd or 35 kd protein (bovine) indicate that both fractions are identical and therefore must be derivatives of the same protein. This N-terminal sequence was determined by Edman reaction, using a gasphase sequencer (Applied Biosystems), to be as follows (X = unknown):

X-Lys-Val-Ala-Ala-Arg-Thr-Phe-Arg-Ser-Phe (SEQ ID NO:10)

Chromatography of rat 33 kd, 35 kd and 250 kd gel eluted proteins resulted in a very similar peak pattern compared to bovine material, with minor changes in the retention time of the protein peak II of interest. Again, this peak migrated in an SDS-PAGE gel at 33 kd and has a similar, however not identical, N-terminal amino acid sequence compared to the bovine peak. The rat N-terminal sequence (SEQ ID NO:4) was determined, and is shown in Figure 16. Also shown in Figure 16 are the partial sequences of its predicted mRNA (SEQ ID NO:5), and a complementary DNA (SEQ ID NO:6) which can be used as a hybridization probe for cloning the NI-35 (or NI-250, see infra) gene.

The N-terminus of peak II derived from rat 35 kd or 250 kd protein fractions was determined to be identical to each other. This result therefore shows that the 250 kd protein is a complex containing the 35 kd protein.

Endoproteinase Lys-C digestion of the rat 35 kD HPLC-purified protein resulted in several fragments which were separated by HPLC. A partial sequence of 17 amino acids (SEQ ID NO:7) was determined and is shown in Figure 17, along with its predicted partial mRNA sequence (SEQ ID NO:8), and a DNA (SEQ ID NO:9) which can be used as a hybridization probe in cloning.

No significant homologies for the amino acid sequences represented by SEQ ID NO:4, NO:7 or NO:10 were found in the EMBL databank.

Of interest is our finding that neither the 35 kd nor the 250 kd neurite growth inhibitory factor binds to peanut agglutinin.

9. ISOLATION AND CHARACTERIZATION OF HUMAN NEURITE GROWTH INHIBITORY FACTORS

Human CNS myelin was prepared by the same procedure described by Caroni and Schwab (1988, J. Cell Biol. 106:1281-1288). Human material was derived from brain stem and spinal cord. CNS crude myelin was extracted with 2% SDS, pelleted at 45,000 rpm, and the supernatant was precipitated with ice-cold acetone in order to remove the SDS. The precipitated material was redissolved in 20 mM Hepes, pH 7.4 (medium A) containing 2.5% cholate. To this material, phosphatidylcholine dissolved in medium A with 2.5% cholate (ratio 10:1 of lipid: protein) was added, and liposomes were formed on a Sephadex G50 column by the procedure described by Brunner et al. (1978, J. Biol. Chem. 250:7500-7546). The activity of these liposomes was tested in the 3T3 spreading assay (Caroni and Schwab, 1988, Neuron 1:85-96 and Caroni and Schwab, 1988, J. Cell Biol. 106:1281-1288). Results are presented in Table VI.

TABLE VI
THE INHIBITORY ACTIVITY IN HUMAN CNS MYELIN

5	<u>Substrate</u>	<u>3T3 Spreading</u>
	Tissue culture plastic	1,645 +/- 287
	Liposomes with myelin protein	498 +/- 69

10 As shown by the results in Table VI, extracts of human
CNS myelin had inhibitory activity when tested in the
fibroblast spreading assay.

15 Gel-eluted human 35 kD and 250 kD protein was
subjected to HPLC chromatography, resulting in protein peak
patterns that were very similar to each other, including the
common peak shared by bovine and rat 35 kD and 250 kD
proteins. The amino acid composition of the human 35 kD
protein HPLC-eluted peak was similar but not identical to
that of bovine and rat NI-35. The approximate amino acid
20 composition of the human NI-35 was determined to be as
follows:

25	Ala: 1.3
	Cys: 1.0
	Asp: 0.9
	Glu: 1.0
	Phe: 0.8
	Gly: 0.5
	His: 1.0
	Ile: 0.7
30	Lys: 1.0
	Leu: 0.7
	Met: 0.5
	Asn: 1.2
	Pro: 0.5
35	Gln: 1.0

The N-terminus of the human protein was blocked, but Endoproteinase-Lys C digestion result d in an HPLC peak pattern similar to that of the rat protein. A preliminary
5 internal peptide sequence of NI-35, after Endoproteinase-Lys C digestion, was obtained (Xaa = unknown):

Ala-Val-His-Phe-Asn-Gly-Ala-Phe-Glu-Xaa-Ser-His-Thr
-Val-Tyr-Gln-Asp-Xaa-Gly (SEQ ID NO:11).

10 10. MALIGNANT POTENTIAL IN VIVO OF HUMAN GLIOBLASTOMA
CELL LINES CORRELATES WITH THEIR IN VITRO
SENSITIVITY TO METALLOPROTEASE INHIBITORS

15 Metalloprotease inhibitors were found to impair human glioblastoma cell spreading on CNS myelin in vitro. Our experiments have shown that there exists a correlation
between in vivo invasiveness and lethality of human
glioblastoma cells and their infiltrative behavior in vitro
as measured by their spreading on CNS myelin or the
sensitivity of such spreading to metalloprotease inhibitors.

20 Human glioblastoma cell lines were obtained by isolation of tumor cells from a single patient, and
subcloning. Eight cell lines were obtained, coded SKI A, B,
C, D, E, G, H and I. Each cell line was observed to possess
unique properties, rendering it distinct from the other cell
lines. The cell lines were tested in vitro for the
25 following:

- (a) spreading on PLYS, or CNS myelin in serum-free medium (MEM α)
- (b) inhibition of cell spreading on CNS myelin by metalloprotease blockers (o-phenanthroline, cbz-Gly-Phe-NH₂, cbz-Tyr-Tyr-OH)
30
- (c) production of plasminogen activator (PA) using a casein-overlay method

The results are shown in Table VII.

TABLE VII
IN VITRO ASSAYS OF HUMAN
GLIOBLASTOMA CELL LINES *

	<u>Cell line</u>	<u>Spreading on CNS myelin</u>	<u>Sensitivity to metallopro- tease blockers</u>	<u>Production of Plasminogen Activator</u>
5	A	+(±)	+	+++
10	B	+	+	-
	C	+(±)	++	++
	D	(±)	+	++
	E	+++	++	-
15	G	(±)	+	(±)
	H	+	+	+++
	I	(±)	+	++++
20	rat C6 cells	++++	++	-

*Legend:

- ++++: very pronounced spreading, no inhibition by
CNS myelin
25 +: very limited spreading, inhibition

The glioblastoma cell lines were also tested for in vivo invasiveness by intracerebral implantation in nude mice, and survival time was measured. A clear correlation was found between malignant potential in vivo (first animals died in 6 weeks with cell line SKI E; longest survival time was about 5-6 months with cell lines SKI H and I), and infiltrative behavior in vitro (spreading on CNS myelin, sensitivity to metalloprotease blockers). In contrast, the

30

35

production of plasminogen activator was not at all related to high malignancy.

5

11. DEPOSIT OF HYBRIDOMAS

The following hybridomas, producing the indicated monoclonal antibodies, have been deposited with the European Collection of Animal Cell Cultures (ECACC), PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, United Kingdom, and have been assigned the listed accession numbers.

	<u>Hybridoma</u>	<u>Antibody</u>	<u>Accession</u>
	<u>Number</u>		
15	Cell line IN-1	IN-1	88102801
	Cell line IN-2	IN-2	88102802

The present invention is not to be limited in scope by the cell lines deposited or the embodiments disclosed in the examples which are intended as illustrations of a few aspects of the invention and any embodiment which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art and are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

30

35

WHAT IS CLAIMED IS:

1. A substantially pure neurite growth regulatory factor consisting of a molecule characterized by the following properties:
 - (a) a metalloendoprotease activity capable of cleaving the peptide benzyloxycarbonyl-Phe-Ala-Phe-Tyr, which activity is maximally active at a pH in the range of 5.5 to 7.0 and is substantially lost in 1% (w/v) CHAPS;
 - (b) capable of being derived from the plasma membrane of a glioma cell.
2. A fragment or derivative of the factor of claim 1 which substantially retains the metalloendoprotease activity.
3. The factor of claim 1 which is further characterized by the ability to neutralize the inhibitory substrate property of CNS myelin of a higher vertebrate, in which the neutralization is detected by observing the ability of the CNS myelin in the presence of the factor to support neurite outgrowth or fibroblast spreading in vitro.
4. The fragment or derivative of claim 2 which is further characterized by the ability to neutralize the inhibitory substrate property of CNS myelin of a higher vertebrate, in which the neutralization is detected by observing the ability of the CNS myelin in the presence of the factor to support neurite outgrowth or fibroblast spreading in vitro.
5. A method of determining the malignant potential of a cell comprising measuring the metalloprotease activity in vitro of a metalloprotease expressed by the cell, which metalloprotease is capable of neutralizing the inhibitory

substrate property of CNS myelin of a higher vertebrate, in which the neutralization is detected by observing the ability of the CNS myelin in the presence of the
5 metalloprotease to support neurite outgrowth or fibroblast spreading in vitro.

6. A method of determining the malignant potential of a cell comprising measuring the metalloprotease activity in
10 vitro of a metalloprotease expressed by the cell, which metalloprotease is characterized by the following properties:

- 15 (a) a metalloendoprotease activity capable of cleaving the peptide benzyloxycarbonyl-Phe-Ala-Phe-Tyr, which activity is maximally active at a pH in the range of 5.5 to 7.0 and is substantially lost in 1% (w/v) CHAPS; and
- (b) capable of being derived from the plasma membrane of a glioma cell.

20

7. The method according to claim 5 in which the metalloprotease activity is measured by an in vitro peptide substrate assay.

25 8. The method according to claim 6 in which the metalloprotease activity is measured by an in vitro peptide substrate assay.

9. The method according to claim 7 or 8 in which the
30 assay is carried out by determining the concentration of peptide substrate necessary to achieve a defined level of inhibition of spreading by the cell on CNS myelin.

10. The method according to claim 9 in which the
35 peptide substrate is benzyloxycarbonyl-Phe-Ala-Phe-Tyr.

11. The method according to claim 5, 6, 7 or 8 in which the cell is a glial cell.

5 12. The method according to claim 9 in which the cell is a glial cell.

13. A substantially pure human neurite growth inhibitory factor consisting of a human protein having an approximate molecular weight of 35,000 daltons as determined by polyacrylamide gel electrophoresis, and characterized by containing the following amino acid sequence:

10 ...Ala-Val-His-Phe-Asn-Gly-Ala-Phe-Glu-Xaa-Ser-His-Thr
15 -Val-Tyr-Gln-Asp-Xaa-Gly (SEQ ID NO:11)...

14. The factor of claim 13 which is characterized by a nonpermissive substrate property as detected by the ability to inhibit neurite outgrowth or fibroblast spreading
20 in vitro.

15. The factor of claim 14 which is capable of being isolated from human central nervous system myelin.

25 16. A substantially pure neurite growth inhibitory factor consisting of a human protein characterized by:
(a) an approximate molecular weight of 35,000 daltons as determined by polyacrylamide gel electrophoresis;
30 (b) a nonpermissive substrate property as detected by the ability to inhibit neurite outgrowth or fibroblast spreading in vitro; and
(c) an approximate amino acid composition as contained in the following table:

35

5 Ala: 1.3
 Cys: 1.0
 Asp: 0.9
 Glu: 1.0
 Phe: 0.8
 Gly: 0.5
 His: 1.0
 Ile: 0.7
10 Lys: 1.0
 Leu: 0.7
 Met: 0.5
 Asn: 1.2
 Pro: 0.5
15 Gln: 1.0

17. The factor of claim 16 which is further characterized by containing the following amino acid sequence:

20 ...Ala-Val-His-Phe-Asn-Gly-Ala-Phe-Glu-Xaa-Ser-His-Thr
 -Val-Tyr-Gln-Asp-Xaa-Gly (SEQ ID NO:11)...

18. The factor of claim 17 which is capable of being isolated from human central nervous system myelin.

25 19. A fragment or derivative of the factor of claim 16 or 17, characterized by a nonpermissive substrate property as detected by the ability to inhibit neurite outgrowth or fibroblast spreading in vitro.

30 20. A method for diagnosing a malignant tumor in a patient comprising detecting the presence of the factor of claim 1 in a sample from the patient.

35 21. The method according to claim 20 in which the malignant tumor is a glioblastoma.

22. A method for treating a patient with a malignant tumor comprising administering an effective amount t the patient of carbobenzoxy-phenylalanine-alanine-phenylalanine-tyrosine amide (SEQ ID NO:1).

23. The method according to claim 22 in which the malignant tumor is a glioblastoma.

24. A method for treating a patient with damage to the central nervous system comprising administering an effective amount of the factor of claim 1 to the patient.

25. The method according to claim 24 in which the damage is due to a stroke, traumatic injury, or a degenerative disorder of the central nervous system.

26. A method for treating a patient with damage to the central nervous system comprising administering an effective amount of the factor of claim 2 to the patient.

27. The method according to claim 26 in which the damage is due to a stroke, traumatic injury, or a degenerative disorder of the central nervous system.

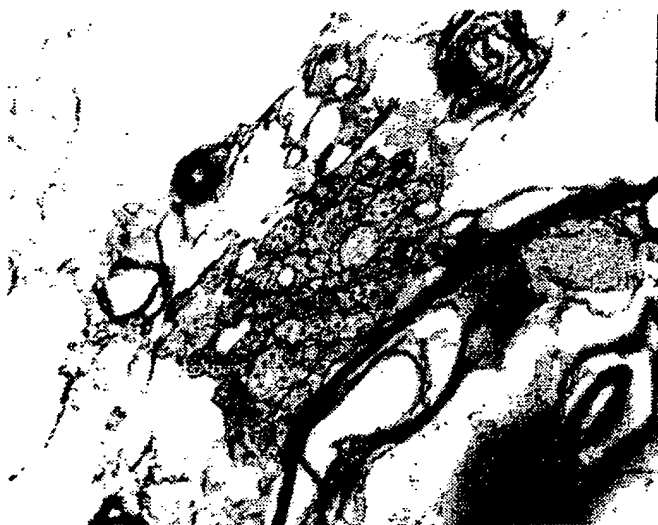


FIG. 1b

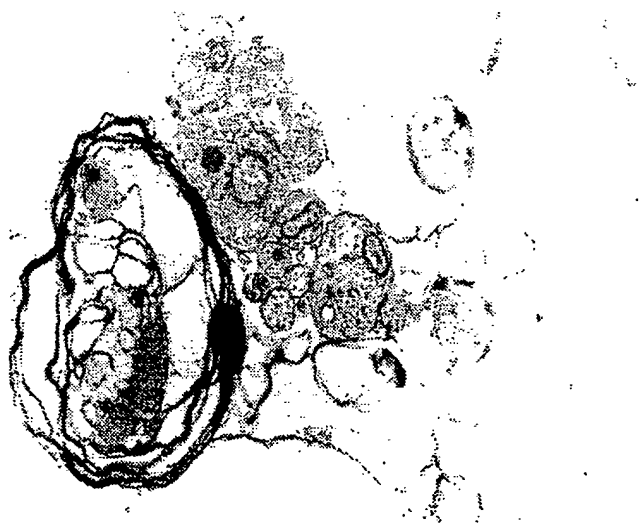
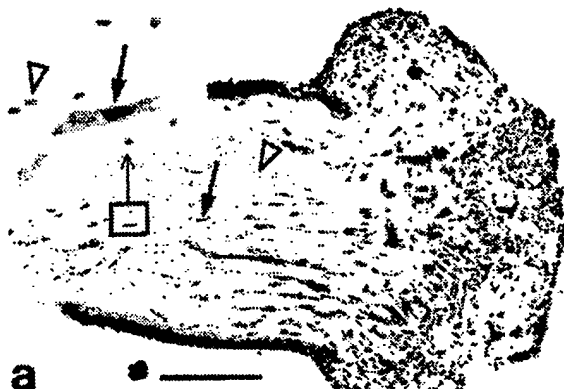
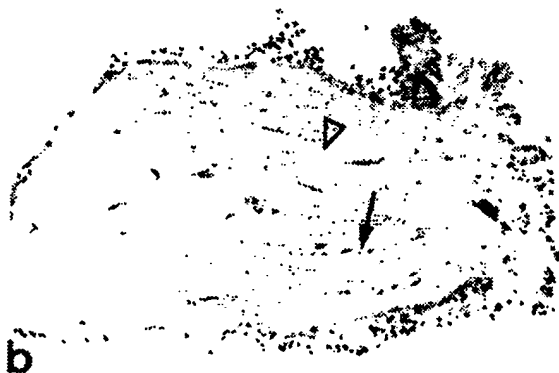


FIG. 1a



a

FIG. 2a



b

FIG. 2b

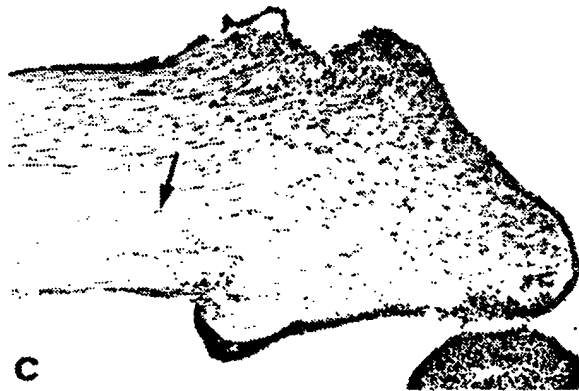


FIG. 2c



FIG. 2d

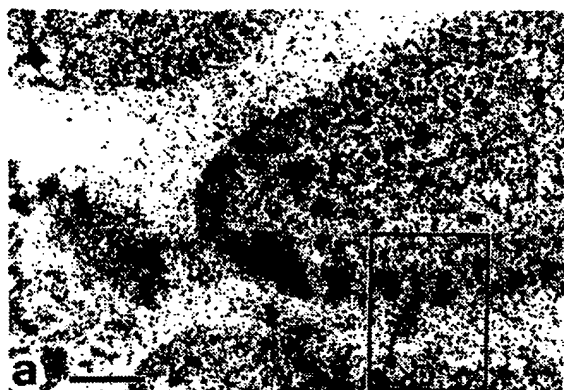


FIG. 3a

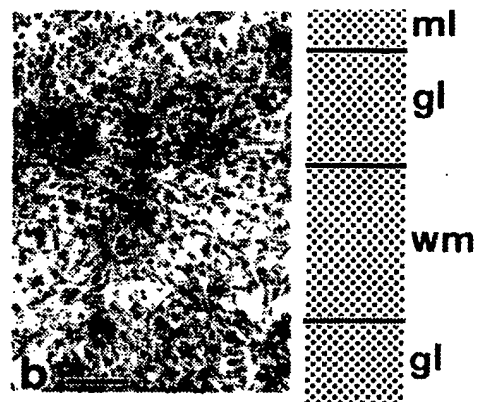


FIG. 3b



FIG. 3c

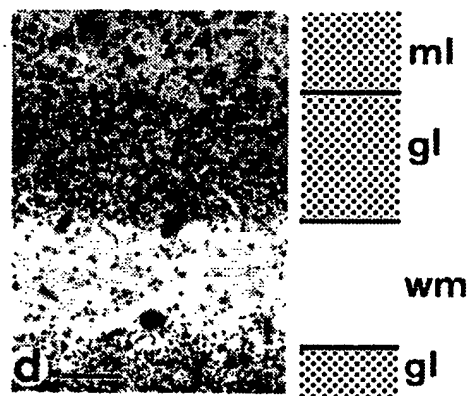


FIG. 3d

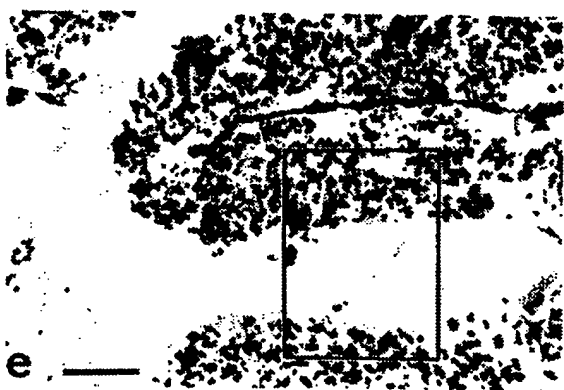


FIG. 3e

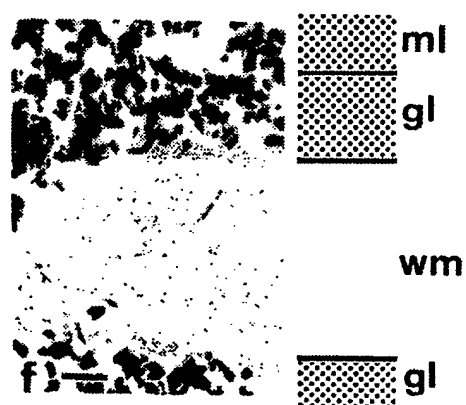


FIG. 3f

5/18

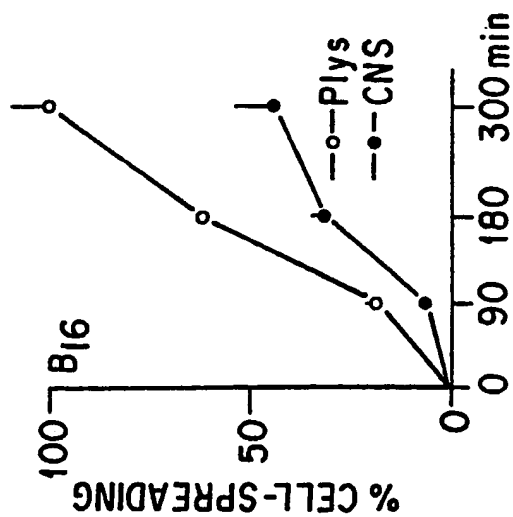


FIG. 4c

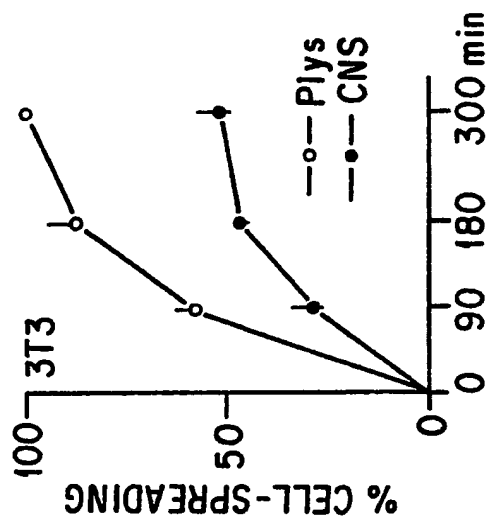


FIG. 4b

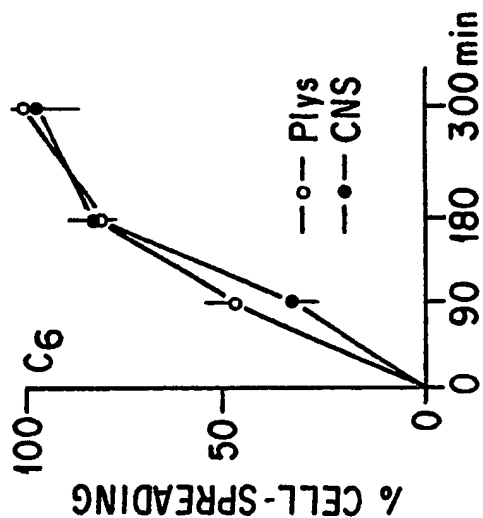


FIG. 4a

6/18

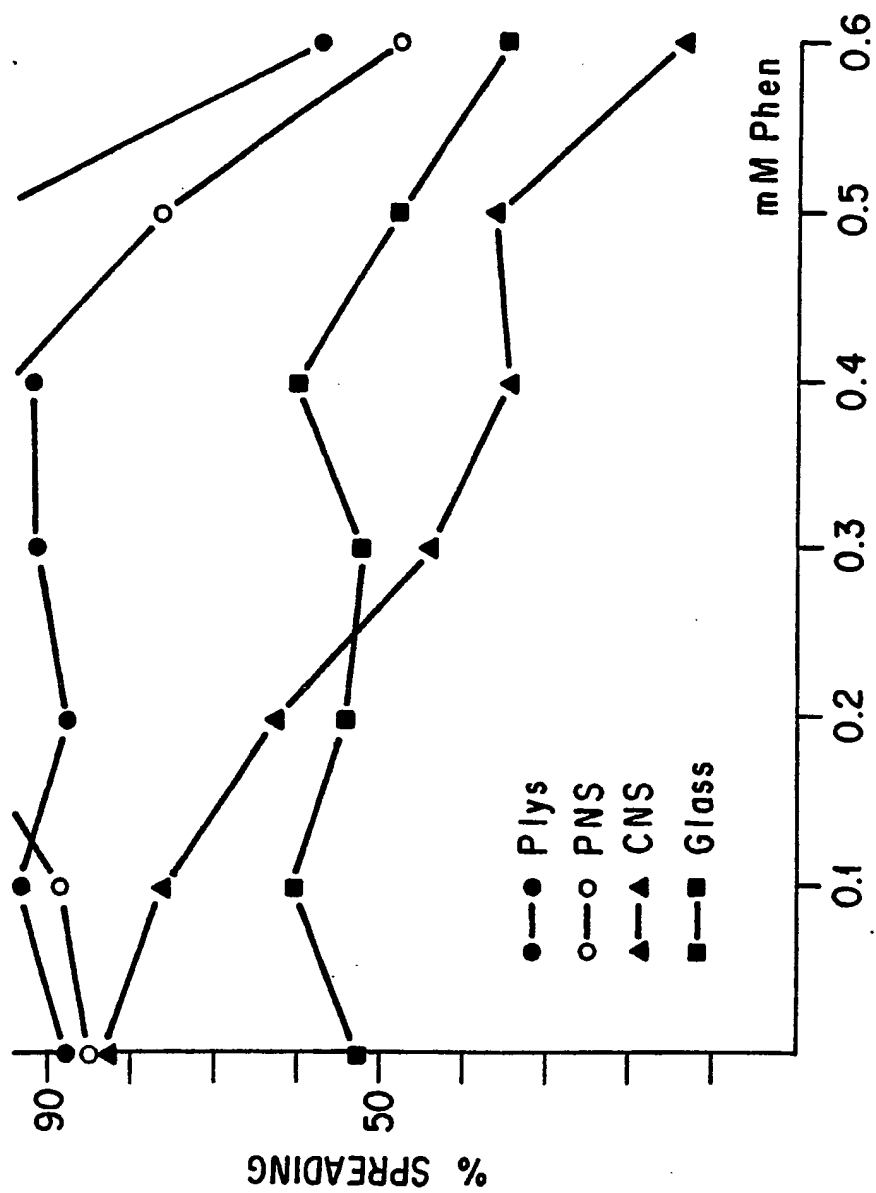


FIG. 5

7/18



FIG. 6b



FIG. 6d

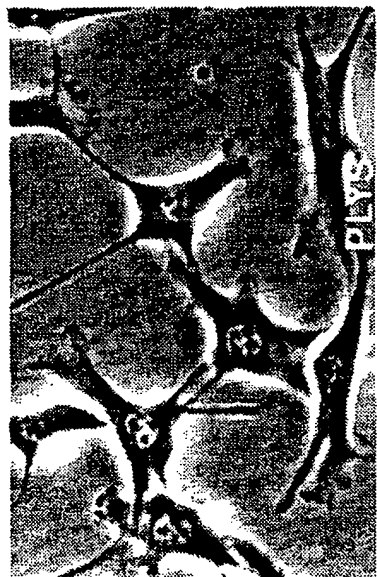


FIG. 6a



FIG. 6c

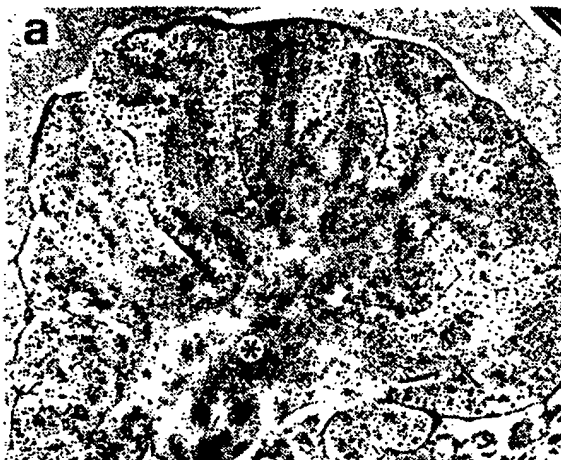


FIG. 7a



FIG. 7b

9/18

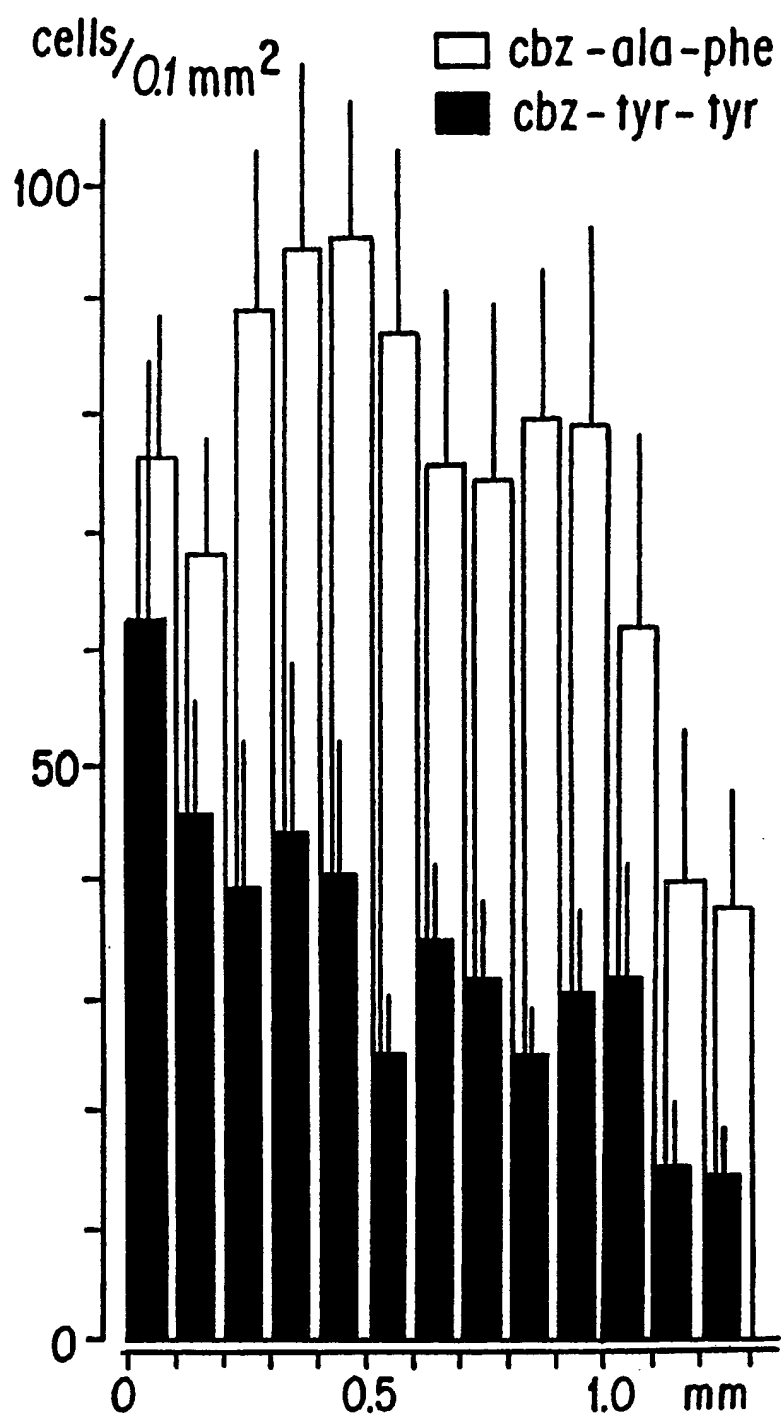


FIG. 8

10/18

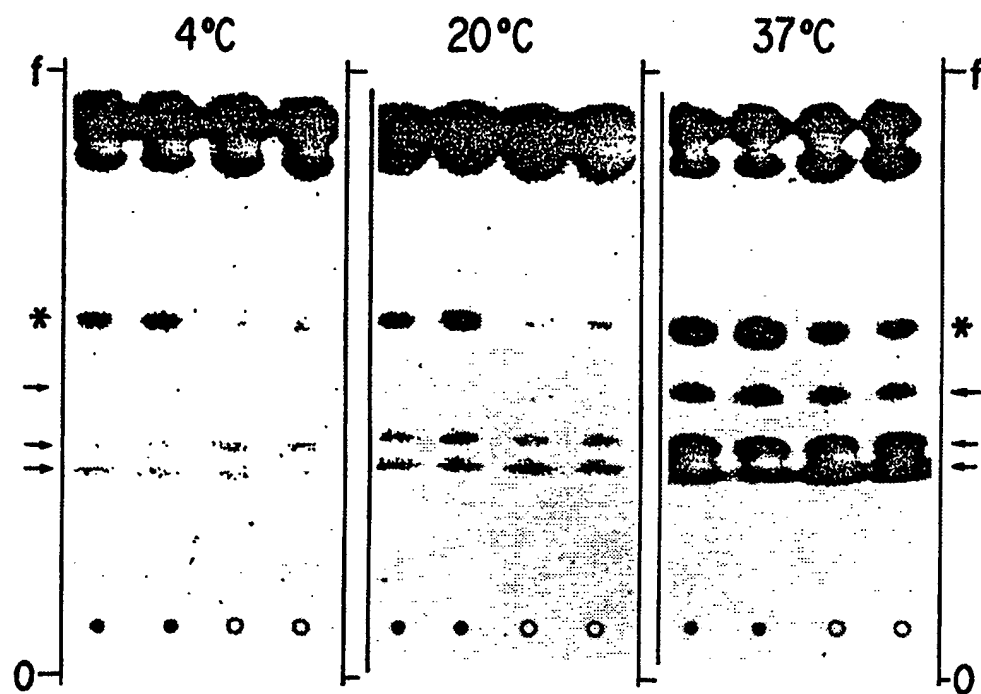


FIG. 9

11/18

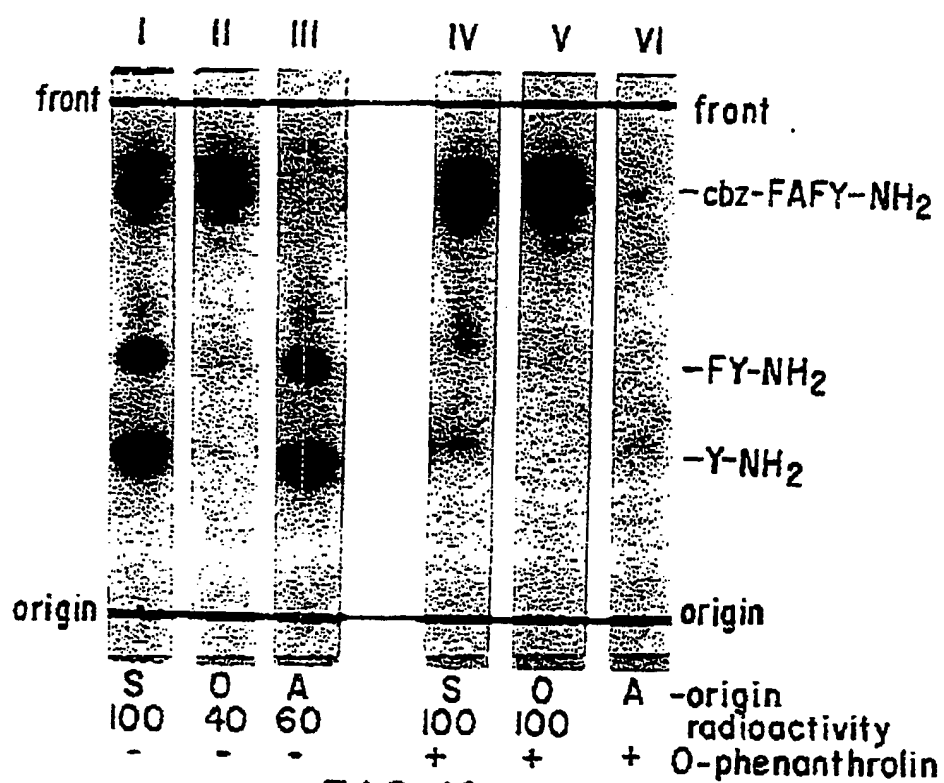


FIG. 10

SUBSTITUTE SHEET
ISA/EP

12/18

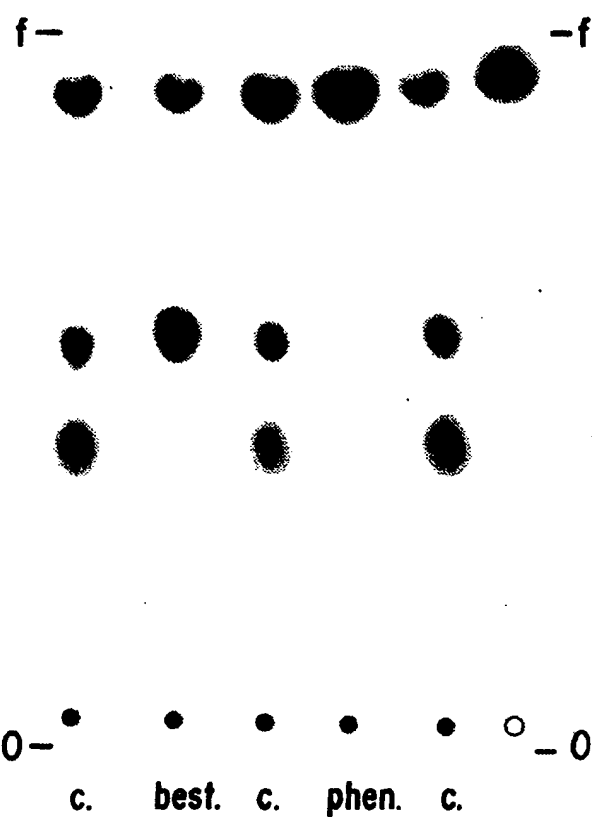
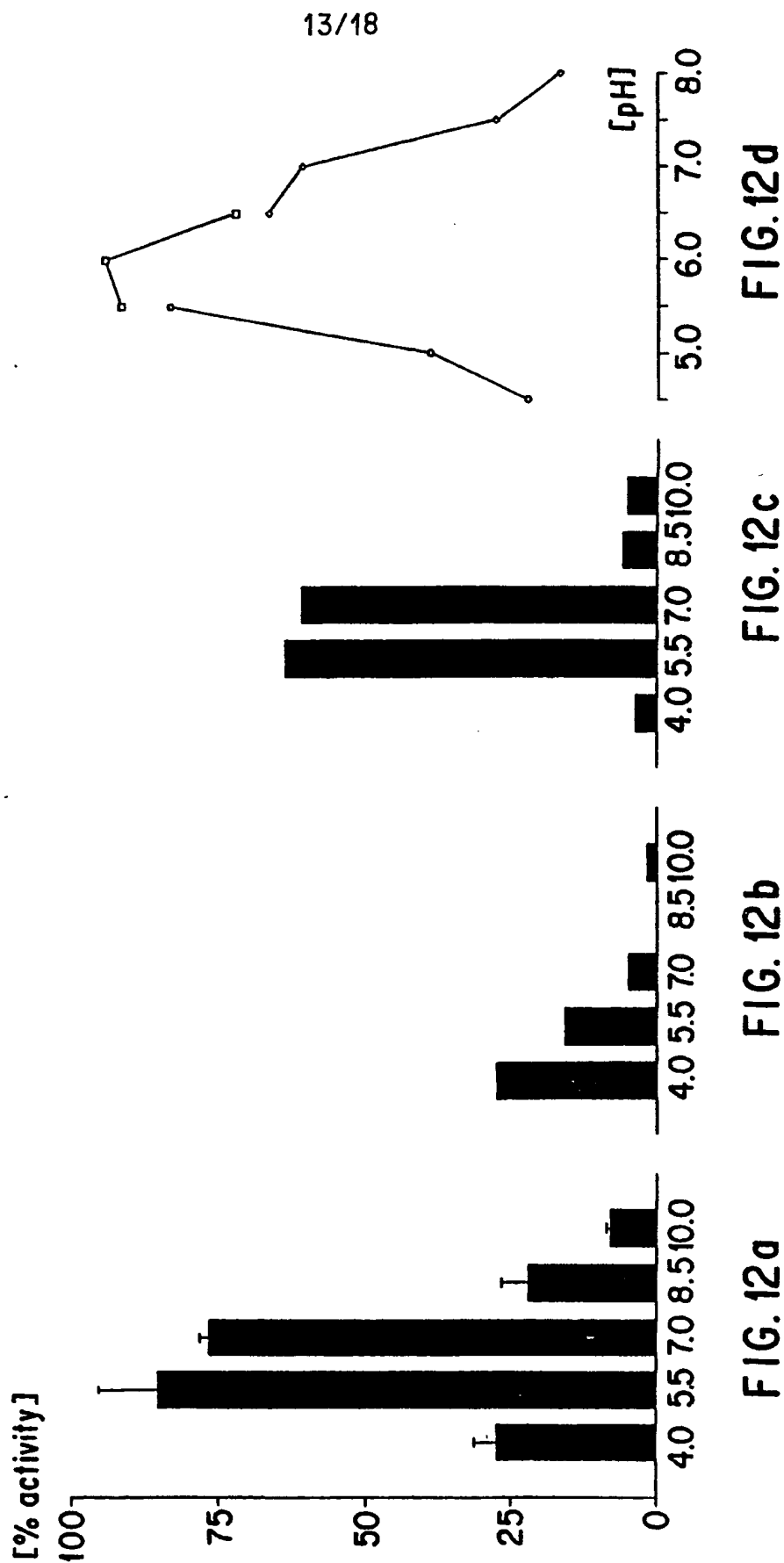
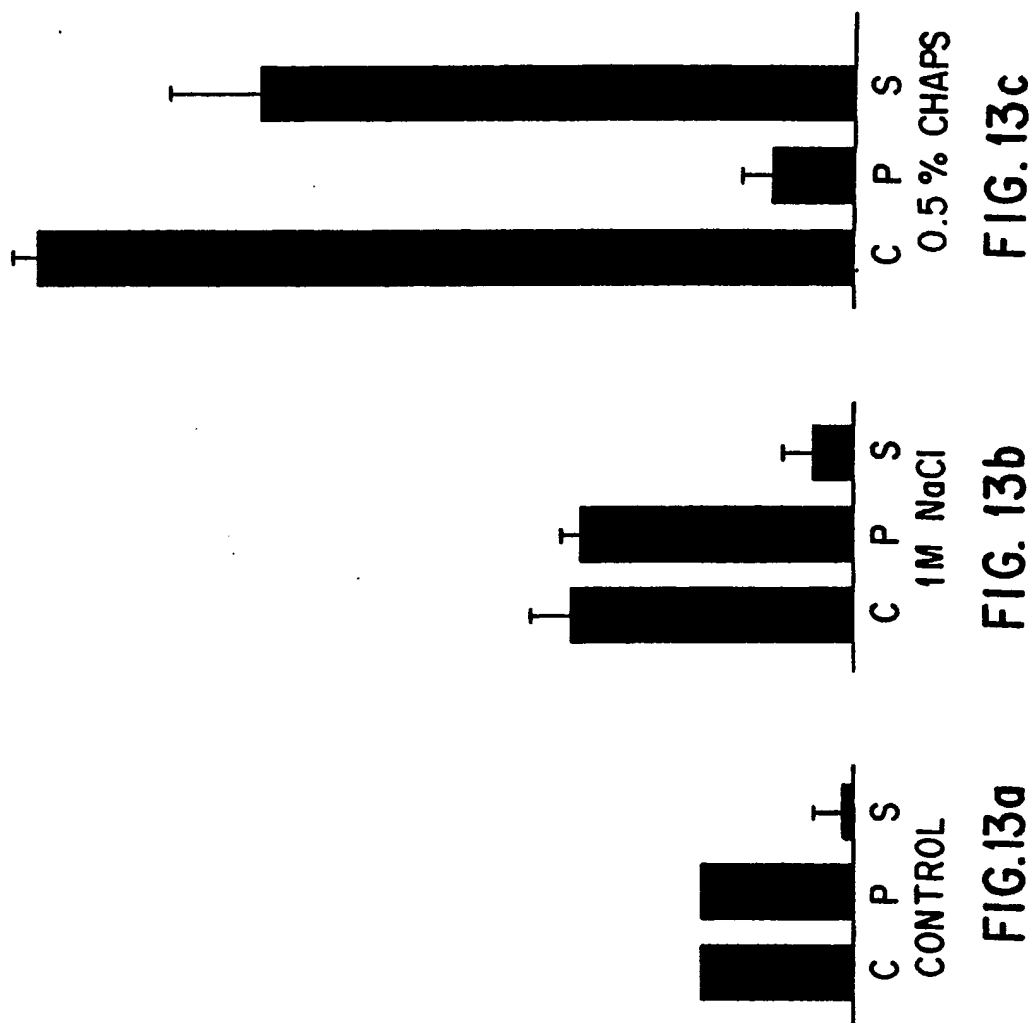


FIG. 11

SUBSTITUTE SHEET



14/18



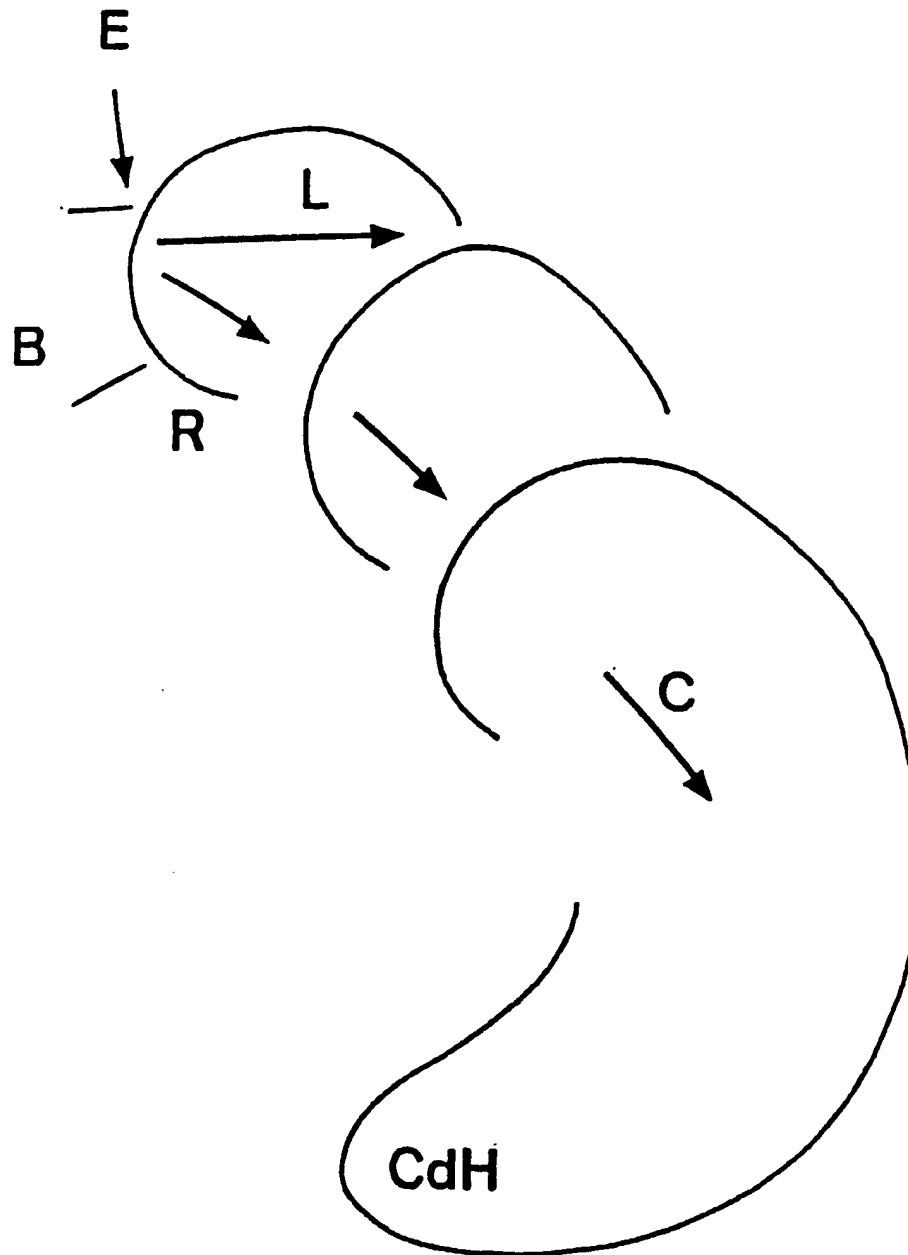


FIG. 14

16 / 18

<u>RAT</u>	<u>BOVINE</u>
A: 1.2	1.2
C: 1.0	1.0
D: 1.0	0.7
E: 1.0	1.0
F: 0.7	1.0
G: 0.5	1.2
H: 1.0	1.0
I: 0.5	0.7
K: 1.0	1.0
L: 0.7	1.0
M: 0.5	0.5
N: 1.2	1.2
P: 0.7	1.0
Q: 1.0	1.2
R: 1.2	1.2
S: 1.0	1.0
T: 1.0	1.0
V: 1.2	1.2
W: 0.7	1.0
Y: 1.0	1.0

FIG. 15

18/18

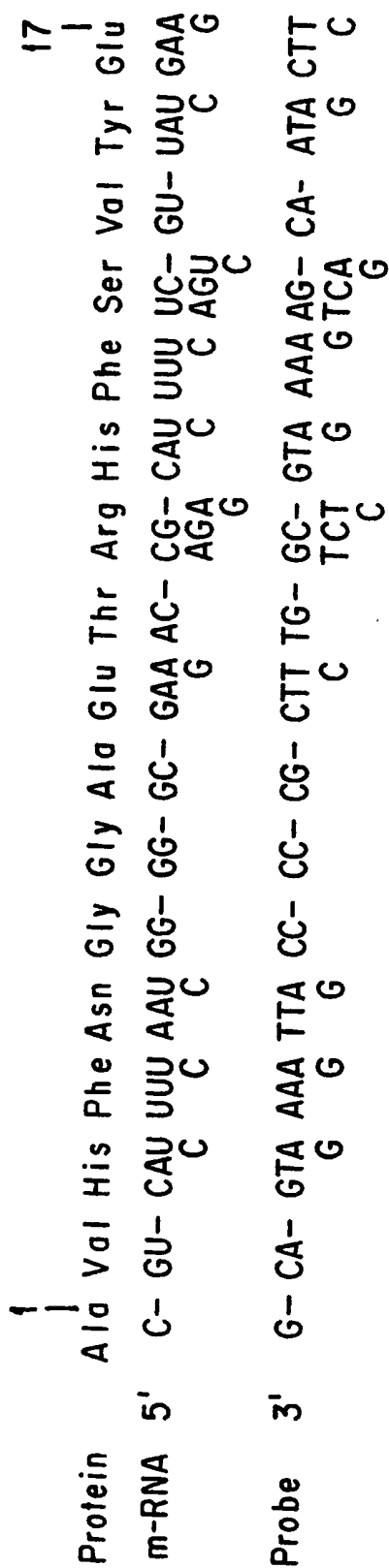


FIG.17